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(54) Title: HUMAN POLYPEPTIDES CAUSING OR LEADING TO THE KILLING OF CELLS INCLUDING LYMPHOID TUMOR CELLS

(57) Abstract: The present invention relates to polypeptide compositions which bind to cell surface epitopes and, in multivalent forms, cause or lead to the killing of cells including lymphoid tumor cells, and in the case of monovalent forms, cause immunosuppression or otherwise inhibit activation of lymphocytes. The invention further relates to nucleic acids encoding the polypeptides, methods for the production of the polypeptides, methods for killing cells, methods for immunosuppressing a patient, pharmaceutical, diagnostic and multivalent compositions and kits comprising the polypeptides and uses of the polypeptides.



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## Human polypeptides causing or leading to the killing of cells including lymphoid tumor cells

### Background of the Invention

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Every mammalian species, which has been studied to date, carries a cluster of genes coding for the so-called major histocompatibility complex (MHC). This tightly linked cluster of genes code for surface antigens, which play a central role in the development of both humoral and cell-mediated immune responses. In humans the products coded for by the MHC are referred to as Human Leukocyte Antigens or HLA. The MHC-genes are organized into regions encoding three classes of molecules, class I to III.

Class I MHC molecules are 45 kD transmembrane glycoproteins, noncovalently associated with another glycoprotein, the 12 kD beta-2 microglobulin (Brown et al., 1993). The latter is not inserted into the cell membrane, and is encoded outside the MHC. Human class I molecules are of three different isotypes, termed HLA-A, -B, and -C, encoded in separate loci. The tissue expression of class I molecules is ubiquitous and codominant. MHC class I molecules present peptide antigens necessary for the activation of cytotoxic T-cells.

Class II MHC molecules are noncovalently associated heterodimers of two transmembrane glycoproteins, the 35 kD  $\alpha$  chain and the 28 kD  $\beta$  chain (Brown et al., 1993). In humans, class II molecules occur as three different isotypes, termed human leukocyte antigen DR (HLA-DR), HLA-DP and HLA-DQ. Polymorphism in DR is restricted to the  $\beta$  chain, whereas both chains are polymorphic in the DP and DQ isotypes. Class II molecules are expressed codominantly, but in contrast to class I, exhibit a restricted tissue distribution: they are present only on the surface of cells of the immune system, for example dendritic cells, macrophages, B lymphocytes, and activated T lymphocytes. They are also expressed on human adrenocortical cells in the zona reticularis of normal adrenal glands and on granulosa-lutein cells in corpora lutea of normal ovaries (Kahoury et al., 1990). Their major biological role is to bind antigenic peptides and present them on the surface of antigen presenting cells (APC) for recognition by CD4 helper T (Th) lymphocytes (Babbitt et al., 1985). MHC class II

molecules can also be expressed on the surface of non-immune system cells, for example, cells that express MHC class II molecules during a pathological inflammatory response. These cells may include synovial cells, endothelial cells, thyroid stromal cells and glial cells.

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Class III MHC molecules are also associated with immune responses, but encode somewhat different products. These include a number of soluble serum proteins, enzymes and proteins like tumor necrosis factor or steroid 21-hydroxylase enzymes. In humans, class III molecules occur as three different isotypes, termed Ca, C2 and Bf (Kuby, 1994).

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Since Th cell activation is a crucial event of the initiation of virtually all immune responses and is mediated through class II molecules, class II MHC offers itself as a target for immunomodulation (Baxevanis et al., 1980; Rosenbaum et al., 1981; Adorini et al., 1988). Besides peptide presentation, class II molecules can transduce various signals that influence the physiology of APC. Such signals arise by the interaction of multiple class II molecules with an antibody or with the antigen receptor of Th cells (Vidovic et al., 1995a; Vidovic et al., 1995b), and can induce B cell activation and immunoglobulin secretion (Cambier et al., 1991; Palacios et al., 1983), cytokine production by monocytes (Palacios, 1985) as well as the up-regulation of co-stimulatory (Nabavi et al., 1992) and cell adhesion molecules (Mourad et al., 1990).

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There is also a set of observations suggesting that class II ligation, under certain conditions, can lead to cell growth arrest or be cytotoxic. Ligation under these conditions is the interaction of a polypeptide with a class II MHC molecule. There is substantial contradiction about the latter effects and their possible mechanisms. Certain authors claim that formation of a complex of class II molecules on B cells leads to growth inhibition (Vaickus et al., 1989; Kabelitz et al., 1989), whereas according to others class II complex formation results in cell death (Vidovic et al., 1995a; Newell et al., 1993; Truman et al., 1994; Truman et al., 1997; Drenou et al., 1999). In certain experimental systems, the phenomenon was observed with resting B cells only (Newell et al., 1993), or in other systems with activated B cells only (Vidovic et al., 1995a; Truman et al., 1994).

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Based on these observations, anti-class II monoclonal antibodies (mAbs) have been envisaged for a number of years as therapeutic candidates. Indeed, this proposal has been supported by the beneficial effect of mouse-derived anti-class II mAbs in a series of animal disease models (Waldor et al., 1983; Jonker et al., 1988; Stevens et al., 1990; Smith et al., 1994; Vidovic & Torral, 1998; Vidovic & Laus, 2000).

Despite these early supporting data, to date no anti-MHC class II mAb of human composition has been described that displays the desired cytotoxic and other biological properties which may include affinity, efficiency of killing and selectivity. Indeed, despite the relative ease by which mouse-derived mAbs may be derived, work using mouse-derived mAbs has demonstrated the difficulty of obtaining an antibody with the desired biological properties. For example, significant and not fully understood differences were observed in the T cell inhibitory capacity of different murine anti-class II mAbs (Naquet et al., 1983). Furthermore, the application of certain mouse-derived mAbs *in vivo* was associated with unexpected side effects, sometimes resulting in death of laboratory primates (Billing et al., 1983; Jonker et al., 1991).

It is generally accepted that mouse-derived mAbs (including chimeric and so-called 'humanized' mAbs) carry an increased risk of generating an adverse immune response (Human anti-murine antibody – HAMA) in patients compared to treatment with a human mAb (for example, Vose et al, 2000; Kashmiri et al., 2001). This risk is potentially increased when treating chronic diseases such as rheumatoid arthritis or multiple sclerosis with any mouse-derived mAb or where regular treatment may be required, for example in the treatment of certain cancers; prolonged exposure of the human immune system to a non-human molecule often leads to the development of an adverse immune reaction. Furthermore, it has proven very difficult to obtain mouse-derived antibodies with the desired specificity or affinity to the desired antigen (Pichla et al. 1997). Such observation may significantly reduce the overall therapeutic effect or advantage provided by mouse-derived mAbs. Examples of disadvantages for mouse-derived mAbs may include the following. First, mouse-derived mAbs may be limited in the medical conditions or length of treatment for a condition for which they are appropriate. Second, the dose rate for mouse-derived mAbs may need to be relatively high in order to compensate for a relatively low affinity or therapeutic effect,

hence making the dose not only more severe but potentially more immunogenic and perhaps dangerous. Third, such restrictions in suitable treatment regimes and high-dose rates requiring high production amounts may significantly add to the cost of treatment and could mean that such a mouse-derived mAb be uneconomical to develop as a commercial therapeutic. Finally, even if a mouse mAb could be identified that displayed the desired specificity or affinity, often these desired features are detrimentally affected during the 'humanization' or 'chimerization' procedures necessary to reduce immunogenic potential (Slavin-Chiorini et al., 1997). Once a mouse-derived mAb has been 'humanized' or chimerized, then it is very difficult to optimize its specificity or affinity.

The art has sought over a number of years for anti-MHC class II mAbs of human composition that show biological properties suitable for use in a pharmaceutical composition for the treatment of humans. Workers in the field have practiced the process steps of first identifying a mouse-derived mAb, and then modifying the structure of this mAb with the aim of improving immunotolerance of this non-human molecule for human patients (for further details, see Jones et al., 1986; Riechmann et al., 1988; Presta, 1992). This modification is typically made using so-called 'humanization' procedures or by fabricating a human-mouse chimeric mAb. Other workers have attempted to identify human antibodies that bind to human antigens having desired properties within natural repertoires of human antibody diversity. For example, by exploring the foetal-tolerance mechanism in pregnant women (Bonagura et al., 1987) or by panning libraries of natural diversities of antibodies (Staubøl-Grøn et al., 1996; Winter et al., 1994). However, to date no anti-MHC class II mAb of human composition has been described that displays the desired biological properties of cytotoxicity, selectivity, specificity, low immunogenicity and affinity.

For therapeutic purposes a polypeptide reacting with many or at least most of the common allelic forms of a human class II MHC molecule would be desirable – e.g., to enable its use in diverse patient populations. Moreover, the candidate polypeptide should be cytotoxic to a wide range of lymphoid tumors, and preferably is cytotoxic by way of a mechanism common to such a range of tumor cells. To allow for a wide range of possible applications, the polypeptide desired should mediate its cytotoxic effect without the dependence on further components of the immune system. For

therapeutic purposes most patients receive for the treatment of e.g. cancer standard chemo- or radiotherapy. Most of these treatments leave the patient immunocompromised. Any additional treatment that relies on an intact immune system is therefore likely to fail. The underlying problem is further demonstrated in humans who suffer from a disease that destroys the immune system, e.g. HIV. Opportunistic infections and malignant transformations are able to escape the immune-surveillance and cause further complications.

### **Summary of the Invention**

One aspect of the present invention relates to a composition including a polypeptide comprising at least one antibody-based antigen-binding domain of human composition with binding specificity for an antigen expressed on the surface of a human cell, wherein treating cells expressing the antigen with a multivalent polypeptide having two or more of said antigen binding domains causes or leads to killing of the cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In certain preferred embodiments the antigen is an MHC antigen, preferably an MHC class II antigen, such as DR/DP/DQ or DR. For instance, in certain preferred embodiments, the subject compositions include a polypeptide comprising at least one antibody-based antigen-binding domain which binds to human HLA DR with a  $K_d$  of 1  $\mu$ M, 100nM, 10nM or even 1nM or less.

Another aspect of the present invention provides a composition including a multivalent polypeptide comprising a plurality of antibody-based antigen-binding domains of human composition with binding specificity for human HLA DR. Treating cells expressing HLA DR with the multivalent polypeptide causes or leads to killing of the cell in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In certain preferred embodiments, the said antigen-binding domains individually bind to the human HLA DR with a  $K_d$  of 1  $\mu$ M, 100nM, 10nM or even 1nM or less. In certain preferred embodiments, the multivalent polypeptide has an  $EC_{50}$  of 100 nM, 10nM or even 1nM or less for killing activated lymphoid cells, transformed cells and/or lymphoid tumor cells.

Still another aspect of the present invention provides a composition including a polypeptide comprising at least one antibody-based antigen-binding domain that binds to human HLA DR with a  $K_d$  of 1  $\mu$ M, 100nM, 10nM or even 1nM or less, the antigen-binding

domain being isolated by a method which includes isolation of human VL and VH domains from a recombinant antibody library by ability to bind to at least one epitope of human HLA DR. Treating a cell expressing HLA DR with a multivalent polypeptide having two or more of the antigen binding domains causes or leads to killing of the cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In certain embodiments, the method for isolating the antigen-binding domain includes the further steps of:

- a. generating a library of variants of at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
- b. isolation of VL and VH domains from the library of variants by ability to bind to human HLA DR with a  $K_d$  of 1  $\mu$ M or less.

In certain preferred embodiments, the composition of the present invention can be characterized as including multivalent polypeptides having an  $EC_{50}$  for killing transformed cells at least 5-fold lower than the  $EC_{50}$  for killing normal cells, and even more preferably at least 10-fold, 100-fold and even 1000-fold less than for killing normal cells.

In certain preferred embodiments, the composition of the present invention are characterized as including multivalent polypeptides having an  $EC_{50}$  for killing activated cells at least 5-fold lower than the  $EC_{50}$  for killing unactivated cells, and even more preferably at least 10-fold, 100-fold and even 1000-fold less than for killing unactivated cells.

In certain preferred embodiments, the composition of the present invention are characterized as including multivalent polypeptides having an  $EC_{50}$  of 50nM or less for killing transformed cells, and even more preferably an  $EC_{50}$  of less than 10nM, 1nM and even 0.1nM. In certain embodiments, the subject multivalent polypeptides have an  $EC_{50}$  for killing activated lymphoid cells, transformed cells and/or lymphoid tumor cells of 100nM, 10nM or even 1nM or less.

In certain embodiments, the subject compositions including multivalent polypeptides selectively kill activated lymphoid cells. For example, such multivalent forms of the subject compositions can be used to kill activated lymphoid cells are lymphoid tumor cells representing a disease selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma,

chronic myeloid leukemia, chronic lymphoid leukemia, and multiple myeloid leukemia. Exemplary activated lymphoid tumor cells which can be killed include Priess, GRANTA-519, KARPAS-422, KARPAS-299, DOHH-2, SR-786, MHH-CALL-4, MN-60, BJAB, RAJI, L-428, HDLM-2, HD-MY-Z, KM-H2, L1236, BONNA-12, HC-1, NALM-1, L-363, EOL-1, LP-1, RPMI-8226, and MHH-PREB-1 cell lines. In certain preferred embodiments, the subject compositions have an  $EC_{50}$  of 100nM or less, and preferably less than 10nM or even 1nM, for killing at least one of B cell lymphoma cells and T cell lymphoma cells selected from the list of KARPAS-422, DOHH-2, SR-7, MHH-CALL-4, MN-60, HD-MY-Z, NALM-1 and LP-1. In certain instances, to effect cell killing, the target cells may require further activation or pre-activation, such as by incubation with Lipopolysaccharide (LPS, 10  $\mu$ g/ml), Interferon-gamma (IFN- $\gamma$ , Roche, 40 ng/ml) and/or phyto-hemagglutinin (PHA, 5  $\mu$ g/ml) to name but a few.

In certain embodiments, the multivalent forms of the subject compositions can be used to kill non-lymphoid cells that express MHC class II molecules.

Certain embodiments, one or more the antigen binding domains of the subject compositions bind to the  $\beta$ -chain of HLA-DR, e.g., the antigen-binding domain binds to the first domain of the  $\beta$ -chain of HLA-DR.

In certain other embodiments, one or more the antigen binding domains of the subject compositions bind to the  $\alpha$ -chain of HLA-DR, e.g., the antigen-binding domain binds to the first domain of the  $\alpha$ -chain of HLA-DR.

In certain preferred embodiments, the the antigen binding domain(s) of the subject compositions bind to one or more HLA-DR types selected from the group consisting of DR1-0101, DR2-15021, DR3-0301, DR4Dw4-0401, DR4Dw10-0402, DR4Dw14-0404, DR6-1302, DR6-1401, DR8-8031, DR9-9012, DRW53-B4\*0101 and DRW52-B3\*0101. In preferred embodiments, the the antigen binding domains of the subject compositions provide broad-DR reactivity, that is, the antigen-binding domain(s) of a given composition binds to epitopes on at least 5 different of said HLA-DR types. In certain embodiments, the antigen binding domain(s) of a polypeptide(s) of the subject compositions binds to a plurality of HLA-DR types as to bind to HLA DR expressing cells for at least 60 percent of the human population, more preferably at least 75 percent, and even more preferably 85 percent of the human population.



In certain embodiments, the antigen-binding domains of the subject compositions include a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

In certain embodiments, the antigen-binding domains of the subject compositions include a combination of HuCAL VH2 and HuCAL VL1, wherein the VH CDR3, VL CDR1 And VL CDR3 is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

In a further preferred embodiment, the antigen-binding domain is modified compared to a parental antigen-binding domain of the present invention by addition, deletion and/or substitution of amino acid residues, while maintaining the properties according to the present invention, or improving one or more of said properties, of said parental antigen-binding domain. This may include, but is not limited to, the modification of a nucleic acid sequence encoding a parental antigen-binding domain for cloning purposes, the modification of CDR regions in order to improve or modify antigen-binding affinity and/or specificity, including the exchange of one or more CDR sequences of a parental antigen-binding domain by corresponding CDR sequences from one or more different antigen-binding domains, and the addition of peptide sequences for detection and/or purification purposes. It is well within the scope of one of ordinary skill in the art to identify positions in a given parental antigen-binding domain where an addition, deletion and/or substitution should occur, to design and pursue the approach to achieve said addition, deletion and/or substitution, and to test or assay whether the modified antigen-binding domain has maintained the properties of, or exhibits one or more improved properties compared to, the parental antigen-binding domain. Furthermore, one of ordinary skill would be able to design approaches where collections or libraries of modified antigen-binding domains are designed, constructed and screened to identify one or more modified antigen-binding domain which have maintained the properties, or exhibit one or

more improved properties compared to the parental antigen-binding domain. In one example, the first amino acid residue of a HuCAL VH domain comprised in any antigen-binding domain or the present invention, which is either E or Q depending on the expression construct, may be exchanged by Q or E, respectively. Preferred regions to  
 5 optimize an antigen-binding domain by designing, constructing and screening collections or libraries of modified antigen-binding domains according to the present invention comprise the CDR regions, and most preferably CDR3 of VH and VL, CDR1 of VL and CDR2 of VH domains.

10 In certain embodiments, the antigen-binding domains includes a combination of HuCAL VH2 and HuCAL Vλ1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and/or

15 wherein the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

wherein each n independently represents any amino acid residue. For instance, the VH CDR3 sequence can be SPRYGAFDY and/or the VL CDR3 sequence can be QSYDLIRH or QSYDMNVH.

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In certain embodiments, the antigen-binding domains of the subject antigen-binding domain competes for antigen binding with an antibody including a combination of HuCAL VH2 and HuCAL Vλ1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

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nnnnRGnFDn

each n independently represents any amino acid residue; and/or

the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

each n independently represents any amino acid residue. For instance, the VH CDR3  
 30 sequence can be SPRYGAFDY and/or the VL CDR3 sequence can be QSYDLIRH or QSYDMNVH.

In certain preferred embodiments, the antigen-binding domain includes a VL CDR1 sequence represented in the general formula

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SGSnnNIGnNYVn

wherein each n independently represents any amino acid residue. For instance, the CDR1 sequence is SGSESNIGNNYVQ.

5 In preferred embodiments, the mechanism of killing by multivalent forms of the subject compositions involves an innate pre-programmed process of said cell. For instance, the killing is non-apoptotic. Killing by the subject compositions can be dependent on the action of non-caspase proteases, and/or killing which cannot be inhibited by zVAD-fmk or zDEVD-fmk.

10 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide including at least a F(ab')<sub>2</sub> antibody fragment or a mini-antibody fragment.

15 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide comprising at least two monovalent antibody fragments selected from Fv, scFv, dsFv and Fab fragments, and further comprises a cross-linking moiety or moieties.

20 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide comprising at least one full antibody selected from the antibodies of classes IgG1, 2a, 2b, 3, 4, IgA, and IgM.

25 In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide is formed prior to binding to said cell.

In certain preferred embodiments, the antibody-based antigen-binding domain is part of a multivalent polypeptide is formed after binding to said cell.

30 In certain preferred embodiments, the antigen binding sites are cross-linked to a polymer.

Another aspect of the present invention provides a nucleic acid comprising a coding sequence for an antigen-binding domain, such as those antigen binding domains described above, or a multivalent polypeptide thereof. For example, in certain embodiments, the nucleic acid includes a coding sequence for a polypeptide  
35 comprising at least one antibody-based antigen-binding domain of human

composition with binding specificity for an antigen expressed on the surface of a human cell, wherein treating cells expressing the antigen with a multivalent form of the polypeptide causes or leads to killing of said cell in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In certain  
5 embodiments, the nucleic acid includes a coding sequence for a polypeptide comprising at least one antibody-based antigen-binding domain which binds to at least one epitope of human HLA DR with a  $K_d$  of 1 $\mu$ M, 100nM, 10nM or even 1nM or less.

In certain embodiments, the nucleic acid includes a coding sequence for a polypeptide  
10 comprising a plurality of antibody-based antigen-binding domains of human composition with binding specificity for human HLA DR, wherein treating a cell expressing HLA DR with the multivalent polypeptide causes or leads to killing of the cell in a manner where neither cytotoxic entities nor immunological mechanisms are needed for killing. In preferred embodiments, the antigen-binding domains individually bind to epitopes on the  
15 human HLA DR with a  $K_d$  of 1 $\mu$ M, 100nM, 10nM or even 1nM or less.

In certain embodiments, the nucleic acid includes a coding sequence for a multivalent polypeptide comprising a plurality of antibody-based antigen-binding domains of human composition with binding specificity for human HLA DR, wherein treating a cell  
20 expressing HLA DR with said multivalent polypeptide causes or leads to killing of said cell in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said cell killing. Preferably, the multivalent polypeptide has an  $EC_{50}$  for killing killing activated lymphoid cells, transformed cells and/or lymphoid tumor cells of 100nM, 10nM or even 1nM or less.

25 Another aspect of the invention provides a vector comprising the coding sequence of any one of the subject nucleic acids, e.g., as described above, and a transcriptional regulatory sequence operably linked thereto.

30 Still another aspect of the present invention provides a host cell harboring at least one subject nucleic acids or the subject vector. Another aspect of the present invention provides a method for the production of a multivalent composition that causes or leads to killing of cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing comprising culturing the host  
35 cells under conditions wherein the nucleic acid is expressed either as a polypeptide

comprising a plurality of antigen binding domains or as a polypeptide comprising at least one antigen binding domains which is subsequently treated to form a multivalent composition.

5 Another aspect of the present invention provides forms of the subject polypeptide or nucleic acid compositions, formulated in a pharmaceutically acceptable carrier and/or diluent. The present invention specifically contemplates the use of such compositions for preparing a pharmaceutical preparation for the treatment of animals, especially humans.

10 Such pharmaceutical compositions can be used for the treatment of conditions involving unwanted cell proliferation, particularly the treatment of a disorder involving transformed cells expressing MHC class II antigens. For instance, the formulations can be used for the treatment of a disorder selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy  
15 cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma, chronic myeloid leukemia, chronic lymphoid leukemia, multiple myeloid leukemia and B cell precursor leukemia.

Such pharmaceutical preparations can be used for the treatment of diseases involving  
20 unwanted activation of immune cells, such as in the treatment of a disorder selected from rheumatoid arthritis, juvenile arthritis, multiple sclerosis, Grave's disease, insulin-dependent diabetes, narcolepsy, psoriasis, systemic lupus erythematosus, ankylosing spondylitis, transplant rejection, graft vs. host disease, Hashimoto's disease, myasthenia gravis, pemphigus vulgaris, glomerulonephritis, thyroiditis, pancreatitis, insulinitis, primary  
25 biliary cirrhosis, irritable bowel disease and Sjogren syndrome.

Another aspect of the present invention provides a diagnostic composition including the polypeptide or nucleic acid compositions of the present invention. In certain  
30 embodiments, the diagnostic composition includes a polypeptide composition and a cross-linking moiety or moieties.

Still another aspect of the present invention provides a method for killing a cell expressing an antigen on the surface of said cell comprising the step of contacting the cell with a multivalent polypeptide composition of the subject invention.

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Another aspect of the invention provides a method to identify patients that can be treated with a multivalent polypeptide composition, formulated in a pharmaceutically acceptable carrier and/or diluent comprising the steps of

- 5                   a. Isolating cells from a patient;
- b. Contacting said cells with the composition; and
- c. Measuring the degree of killing or immunosuppression of said cells.

10           The present invention also provides a kit to identify patients that can be treated with a multivalent polypeptide composition of the present invention, formulated in a pharmaceutically acceptable carrier and/or diluent comprising

- a. a multivalent polypeptide composition; and
- b. Means to measure the degree of killing or immunosuppression of said cells.

15           In certain embodiments, the kit includes a multivalent polypeptide composition, and a cross-linking moiety. In other embodiments, the kit includes

- a. a multivalent polypeptide composition, and
- b. a detectable moiety or moieties, and
- c. reagents and/or solutions to effect and/or detect binding of (i) to an
- 20                   antigen.

Another aspect of the present invention provides a cytotoxic composition comprising a multivalent polypeptide composition operably linked to a cytotoxic agent.

25           Still another aspect of the invention provides an immunogenic composition comprising a multivalent polypeptide composition operably linked to an immunogenic agent.

Another aspect of the present invention provides a method to kill a cell comprising contacting the cell with a multivalent polypeptide composition operably linked a cytotoxic or immunogenic agent.

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Another aspect of the invention provides a method for treating a human to reduce the severity of disorder involving unwanted proliferation/activation of cells expressing the human  $\beta$ -chain of HLA DR, comprising administering to the patient a a multivalent polypeptidepolypeptide of the present invention. In certain embodiments, the disorder

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involves unwanted proliferation/activation of lymphoid cells, e.g., selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma, chronic myeloid leukemia, chronic lymphoid leukemia, multiple myeloid leukemia and B cell precursor leukemia.

Another aspect of the invention provides a use of a multivalent polypeptide composition operably linked a cytotoxic or immunogenic agent for preparing a pharmaceutical preparation for the treatment of animals

10

According to a preferred embodiment, the polypeptide is directed to a lymphoid cell or a non-lymphoid cell that expresses MHC class II molecules. The latter type of cells occur for example at pathological sites of inflammation and/or autoimmune diseases, e.g. synovial cells, endothelial cells, thyroid stromal cells and glial cells, or it may also comprise genetically altered cells capable of expressing MHC class II molecules.

15

Preferably, the polypeptide is directed to lymphoid tumor cells. More preferred are lymphoid tumor cells that represent a disease selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia and B cell precursor leukemia. Most preferred are lymphoid tumor cells from a cell line taken from the list of GRANTA-519, PRIESS, KARPAS-422, DOHH-2, MHH-CALL-4, MN-60, BJAB, L-428, BONNA-12, EOL-1, MHH-PREB-1 and MHH-CALL-2 cell lines.

20

In certain embodiments, the polypeptide binds to at least one epitope in the alpha-chain of an HLA-DR molecule. In such embodiments, the polypeptide preferably binds to at least one epitope in the first domain of the alpha-chain of HLA-DR, the first domain being the N-terminal domain of the chain. For instance, the polypeptide can be selected to bind to at least one epitope within the alpha-helix ranging from Glu<sup>55</sup> to Tyr<sup>79</sup> of the alpha-chain of HLA-DR.

25

30

In other embodiments, the polypeptide binds to at least one epitope in the beta-chain of an HLA-DR molecule. Preferably, the polypeptide binds to at least one epitope in the first domain of the beta-chain of HLA-DR, the first domain being the N-terminal domain of the chain.

35

In certain embodiments, the mechanism of killing a target cell induced by the polypeptide involves an innate pre-programmed process of said cell. Preferably, the polypeptide induces a killing mechanism, which is not an apoptotic cell death process.

5

In a preferred embodiment the polypeptide induces a killing mechanism which is dependent on the action of proteases other than caspases, e.g., is a caspase-independent mechanism.

- 10 In a further embodiment the multivalent composition comprises at least one full antibody which is selected from classes IgG1, 2a, 2b, 3, 4, IgA, and IgM.

In a further embodiment the multivalent composition comprises at least one of a F(ab')<sub>2</sub> antibody fragment or mini-antibody fragment.

15

In a preferred embodiment the multivalent composition comprises at least two monovalent antibody fragments selected from Fv, scFv, dsFv and Fab fragments, and further comprises a cross-linking moiety or moieties.

- 20 The present invention also provides a composition including a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for human HLA DR wherein binding of said polypeptide to said epitope causes or leads to suppression of the immune response and wherein said antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination
- 25 is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

30

Another immunosuppressive composition of the present invention includes a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for a human MHC class II antigen with a K<sub>d</sub> of 1μM, 100nM, 10nM or even 1nM or less, wherein treating cells expressing MHC class II antigen with the polypeptide causes or



leads to suppression of the immune response, e.g., preferably with an IC<sub>50</sub> of 1 μM, 100nM, 10nM or even 1nM or less.

5 Another immunosuppressive composition of the present invention includes a polypeptide comprising at least one antibody-based antigen-binding domain of human composition with a binding specificity for a human MHC class II antigen with a K<sub>d</sub> of 1 μM, 100nM, 10nM or even 1nM or less, the antigen-binding domain being isolated by a method which includes isolation of human VL and VH domains from a recombinant antibody display library by ability to bind to human MHC class II antigen, wherein treating cells that  
10 express MHC class II with said polypeptide causes or leads to suppression of the immune response.

The subject immunosuppressive compositions can be generated using the antigen-binding domain isolated by the further steps of:

- 15           a. generating a library of mutations at least one of the CDR1, CDR2 and CDR3 domains of one or both of the VL and VH domains, and  
            b. isolation of VL and VH domains from the library of variants by ability to bind to human MHC class II antigen with a K<sub>d</sub> of 1 μM or less.

20 In preferred embodiments, the antigen binding domains of the immunosuppressive composition binds to HLA-DR, and preferably to the β-chain of HLA-DR, and even more preferably to the first domain of the β-chain of HLA-DR.

In certain preferred embodiments, the immunosuppressive composition have an IC<sub>50</sub> for  
25 suppressing the immune response of 1 μM, 100nM, 10nM or even 1nM or less.

In certain preferred embodiments, the immunosuppressive composition have an IC<sub>50</sub> for inhibiting of IL-2 secretion of 1 μM, 100nM, 10nM or even 1nM or less.

30 In certain preferred embodiments, the immunosuppressive composition have an IC<sub>50</sub> for inhibiting of T cell proliferation of 1 μM, 100nM, 10nM or even 1nM or less.

In certain preferred embodiments, the immunosuppressive composition have antigen-binding domain that bind to an epitope of one or more HLA-DR types selected from the  
35 group consisting of DR1-0101, DR2-15021, DR3-0301, DR4Dw4-0401, DR4Dw10-0402,

DR4Dw14-0404, DR6-1302, DR6-1401, DR8-8031, DR9-9012, DRw53-B4\*0101 and DRw52-B3\*0101, and in preferred embodiments, the antigen-binding domain binds to at least 5 different of said HLA-DR types (e.g., are pan-DR)

- 5 In certain embodiments, the immunosuppressive composition have antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-  
10 GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

In certain embodiments, the immunosuppressive composition have antigen-binding domain includes a combination of HuCAL VH2 and HuCAL VA1, wherein the VH CDR3  
15 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and

wherein the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

20 wherein each n independently represents any amino acid residue.

For instance, the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.

In certain embodiments, the immunosuppressive composition the antigen-binding  
25 domain competes with antigen binding by an antibody having a VH CDR3 sequence represented by the general formula

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and

a VL CDR3 sequence represented by the general formula

30 QSYDnnnn

wherein each n independently represents any amino acid residue.

In certain embodiments, the immunosuppressive composition the antigen-binding domain includes a VL CDR1 sequence represented in the general formula

35 SGSnnNIGnNYVn

wherein each n independently represents any amino acid residue. For example, the CDR1 sequence is SGSESNIGNNYVQ.

5 In certain embodiments, the subject immunosuppressive compositions suppress the immune response by one or more of (a) down-regulation of expression of the antigen to which the polypeptide binds; or (b) *inhibiting of the interaction between said cell and other cells*, wherein said interaction would normally lead to an immune response.

10 Another aspect of the present invention provides nucleic acids which including a coding sequence for an immunosuppressive polypeptide of the present invention. In certain embodiments, the nucleic acid can be provided as part of a vector, e.g., including the coding sequence and a *transcriptional regulatory sequence operably linked thereto*. The nucleic acid and vectors of the present invention can be provided as part of a host cell, e.g., which can be used to to produce an immunosuppressive composition.

15 Another aspect of the present invention provides a method for suppressing activation and/or proliferation of a lymphocyte, comprising contacting the cell with an immunosuppressive polypeptide of the present invention.

20 The present invention also provides a pharmaceutical preparation comprising the a polypeptide including an antibody-based antigen-binding domain with a binding specificity for a human MHC class II antigen with a  $K_d$  of  $1\mu\text{M}$  or less, e.g., in an amount sufficient to suppress an immune response in an animal, inhibit IL-2 secretion in an animal, and/or inhibit T cell proliferation in an animal.

25 Another aspect of the present invention relates to the use of a polypeptide including an antibody-based antigen-binding domain with a binding specificity for a human MHC class II antigen with a  $K_d$  of  $1\mu\text{M}$  or less, for the preparation of a pharmaceutical composition for the treatment of animals, such as where said animals are human.

30 The subject immunosuppressive pharmaceutical preparations can be used for suppressing IL-2 secretion by a cell of the immune system. For example, these preparations can be administered to the patient in an effective amount to reduce the level of immunological responsiveness in the patient.

35

Still another aspect of the present invention provides a method for suppressing IL-2 secretion by a lymphocyte, comprising contacting the cell with an immunosuppressive polypeptide of the present invention.

- 5 The subject method can be used for immunosuppressing a human, e.g., by administering to the patient an effective amount of an immunosuppressive polypeptide of the present invention to reduce the level of immunological responsiveness.

10 The invention further relates to a diagnostic composition containing at least one polypeptide and/or nucleic acid according to the invention, optionally together with further reagents, such as buffers, for performing the diagnosis.

15 In a preferred embodiment the diagnostic composition contains the polypeptide according to the invention cross-linked by at least one moiety. Such moieties can be for example antibodies recognizing an epitope present on the polypeptide such as the FLAG peptide epitope (Hopp et al., 1988; Knappik and Plückthun, 1994) or bifunctional chemical compounds reacting with a nucleophilic amino acid side chain as present in cysteine or lysine (King et al., 1994). Methods for cross-linking polypeptides are well known to the practitioner of ordinary skill in the art.

20 A diagnostic composition containing at least one nucleic acid and/or variant thereof according to the invention is also contemplated.

25 Furthermore, the present invention relates to a kit comprising at least one polypeptide according to the present invention, and a cross-linking moiety.

30 Additionally, the present invention relates to a kit comprising (i) a polypeptide according to the present invention, (ii) a detectable moiety or moieties, and (iii) reagents and/or solutions to effect and/or detect binding of (i) to an antigen.

The present invention further relates to a multivalent composition comprising at least one polypeptide and comprising at least two antigen binding domains.

35 Still another aspect of the present invention provides a method for conducting a pharmaceutical business comprising:

- (i) isolating one or more antigen-binding domains that bind to antigens expressed on the surface of human cells;
- (ii) generating a multivalent composition comprising a plurality of said antigen-binding domains, which multivalent composition kills with an  $EC_{50}$  of 50nM or less transformed or activated cells where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing.;
- (iii) conducting therapeutic profiling of the multivalent compositions for efficacy and toxicity in animals;
- (iv) preparing a package insert describing the multivalent composition for treatment of proliferative disorders; and
- (v) marketing the multivalent composition for treatment of proliferative disorders.

The present invention also provides a method for conducting a life science business comprising:

- (i) isolating one or more antigen-binding domains that bind to antigens expressed on the surface of human cells;
- (ii) generating a multivalent composition comprising a plurality of said antigen-binding domains, which multivalent composition kills with an  $EC_{50}$  of 50nM or less transformed or activated cells where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing.;
- (iii) licensing, jointly developing or selling, to a third party, the rights for selling the multivalent compositions.

In such embodiments, the the antigen-binding domain can be isolated by a method which includes

- a. isolation of VL and VH domains of human composition from a recombinant antibody display library by ability to bind to epitopes of HLA DR,
- b. generating a library of variants at least one of the CDR1, CDR2 and CDR3 domains of one or both of the VL and VH domains, and
- c. isolation of VL and VH domains from the library of variants by ability to epitopes of HLA DR with a  $K_d$  of 1 $\mu$ M or less.

Another business method contemplated by the present invention includes:

- (i) isolating one or more antigen-binding domains that bind to MHC class II expressed on the surface of human cells with a  $K_d$  of 1 $\mu$ M or less;

- (ii) generating a composition comprising said antigen-binding domains, which composition is immunosuppressant with an  $IC_{50}$  of 100nM or less;
- (iii) conducting therapeutic profiling of the multivalent compositions for efficacy and toxicity in animals;
- 5 (iv) preparing a package insert describing the use of the composition for immunosuppression therapy; and
- (v) marketing the multivalent composition for use as an immunosuppressant.

10 The present invention also provides a method for conducting a life science business comprising:

- (i) isolating one or more antigen-binding domains that bind to MHC class II expressed on the surface of human cells with a  $K_d$  of 1 $\mu$ M or less;
- (ii) generating a composition comprising said antigen-binding domains, which composition is immunosuppressant with an  $IC_{50}$  of 100nM or less;
- 15 (iii) licensing, jointly developing or selling, to a third party, the rights for selling the compositions.

As used herein, the term "peptide" relates to molecules consisting of one or more chains of multiple, i. e. two or more, amino acids linked via peptide bonds.

20

The term "protein" refers to peptides where at least part of the peptide has or is able to acquire a defined three-dimensional arrangement by forming secondary, tertiary, or quaternary structures within and/or between its peptide chain(s). This definition comprises proteins such as naturally occurring or at least partially artificial proteins, as well as fragments or domains of whole proteins, as long as these fragments or domains are able to acquire a defined three-dimensional arrangement as described above.

25

The term "polypeptide" is used interchangeably to refer to peptides and/or proteins. Moreover, the terms "polypeptide" and "protein", as the context will admit, include multi-chain protein complexes, such as immunoglobulin polypeptides having separate heavy and light chains.

30

In this context, "polypeptide comprising at least one antibody-based antigen-binding domain" refers to an immunoglobulin (or antibody) or to a fragment thereof. The term

35

"fragment", with respect to antibody domains and the like, refers to a fragment of an immunoglobulin which retains the antigen-binding moiety of an immunoglobulin. Functional immunoglobulin fragments according to the present invention may be Fv (Skerra and Plückthun, 1988), scFv (Bird et al., 1988; Huston et al., 1988), disulfide-linked Fv (Glockshuber et al., 1992; Brinkmann et al., 1993), Fab, F(ab')<sub>2</sub> fragments or other fragments well-known to the practitioner skilled in the art, which comprise the variable domains of an immunoglobulin or functional immunoglobulin fragment.

Examples of polypeptides consisting of one chain are single-chain Fv antibody fragments, and examples for polypeptides consisting of multiple chains are Fab antibody fragments.

The term "antibody" as used herein, unless indicated otherwise, is used broadly to refer to both antibody molecules and a variety of antibody derived molecules. Such antibody derived molecules comprise at least one variable region (either a heavy chain or light chain variable region) and include such fragments as described above, as well as individual antibody light chains, individual antibody heavy chains, chimeric fusions between antibody chains and other molecules, and the like.

The "antigen-binding site" of an immunoglobulin molecule refers to that portion of the molecule that is necessary for binding specifically to an antigen. An antigen binding site preferably binds to an antigen with a K<sub>d</sub> of 1 μM or less, and more preferably less than 100 nM, 10 nM or even 1 nM in certain instances. Binding specifically to an antigen is intended to include binding to the antigen which significantly higher affinity than binding to any other antigen.

The antigen binding site is formed by amino acid residues of the N-terminal variable ("V") regions of the heavy ("H") and light ("L") chains. Three highly divergent stretches within the V regions of the heavy and light chains are referred to as "hypervariable regions" which are interposed between more conserved flanking stretches known as "framework regions," or "FRs". Thus the term "FR" refers to amino acid sequences which are naturally found between and adjacent to hypervariable regions in immunoglobulins. In an antibody molecule, the three hypervariable regions of a light chain and the three hypervariable regions of a heavy chain are disposed relative to

each other in three dimensional space to form an antigen-binding surface. The antigen-binding surface is complementary to the three-dimensional surface of a bound antigen, and the three hypervariable regions of each of the heavy and light chains are referred to as "complementarity-determining regions," or "CDRs."

5

For the purposes of this application, "valent" refers to the number of antigen binding sites the subject polypeptide possess. Thus, a bivalent polypeptide refers to a polypeptide with two binding sites. The term "multivalent polypeptide" encompasses bivalent, trivalent, tetravalent, etc. forms of the polypeptide.

10

As used herein, a "multivalent composition" means a composition comprising a polypeptide having at least two of said antigen-binding domains, e.g., a multivalent polypeptide. Preferably, said at least two antigen-binding domains are in close proximity so as to mimic the structural arrangement relative to each other of binding sites comprised in a full immunoglobulin molecule. Examples for multivalent compositions are full immunoglobulin molecules (e.g. IgG, IgA or IgM molecules) or multivalent fragments thereof (e.g. F(ab')<sub>2</sub>). Additionally, multivalent compositions of higher valencies may be formed from two or more multivalent compositions (e.g. two or more full immunoglobulin molecules), e.g. by cross-linking. Multivalent compositions, however, may be formed as well from two or more monovalent immunoglobulin fragments, e.g. by self-association as in mini-antibodies, or by cross-linking.

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Accordingly, an "antibody-based antigen-binding domain" refers to polypeptide or polypeptides which form an antigen-binding site retaining at least some of the structural features of an antibody, such as at least one CDR sequence. In certain preferred embodiments, antibody-based antigen-binding domain includes sufficient structure to be considered a variable domain, such as three CDR regions and interspersed framework regions. Antibody-based antigen-binding domain can be formed single polypeptide chains corresponding to VH or VL sequences, or by intermolecular or intramolecular association of VH and VL sequences.

The term "recombinant antibody library" describes a variegated library of antigen binding domains. For instance, the term includes a collection of display packages,



e.g., biological particles, which each have (a) genetic information for expressing at least one antigen binding domain on the surface of the particle, and (b) genetic information for providing the particle with the ability to replicate. For instance, the package can display a fusion protein including an antigen binding domain. The antigen binding domain portion of the fusion protein is presented by the display package in a context which permits the antigen binding domain to bind to a target epitope that is contacted with the display package. The display package will generally be derived from a system that allows the sampling of very large variegated antibody libraries. The display package can be, for example, derived from vegetative bacterial cells, bacterial spores, and bacterial viruses.

In an exemplary embodiment of the present invention, the display package is a phage particle which comprises a peptide fusion coat protein that includes the amino acid sequence of a test antigen binding domains. Thus, a library of replicable phage vectors, especially phagemids (as defined herein), encoding a library of peptide fusion coat proteins is generated and used to transform suitable host cells. Phage particles formed from the chimeric protein can be separated by affinity selection based on the ability of the antigen binding site associated with a particular phage particle to specifically bind a target epitope. In a preferred embodiment, each individual phage particle of the library includes a copy of the corresponding phagemid encoding the peptide fusion coat protein displayed on the surface of that package. Exemplary phage for generating the present variegated peptide libraries include M13, f1, fd, If1, I<sub>ke</sub>, Xf, Pf1, Pf3,  $\lambda$ , T4, T7, P2, P4,  $\phi$ X-174, MS2 and f2.

The term "generating a library of variants of at least one of the CDR1, CDR2 and CDR3" refers to a process of generating a library of variant antigen binding sites in which the members of the library differ by one or more changes in CDR sequences, e.g., not FR sequences. Such libraries can be generated by random or semi-random mutagenesis of one or more CDR sequences from a selected antigen binding site.

As used herein, an "antibody-based antigen-binding domain of human composition" preferably means a polypeptide comprising at least an antibody VH domain and an antibody VL domain, wherein a homology search in a database of protein sequences comprising immunoglobulin sequences results for both the VH and the VL domain in

an immunoglobulin domain of human origin as hit with the highest degree of sequence identity. Such a homology search may be a BLAST search, e.g. by accessing sequence databases available through the National Center for Biological Information and performing a "BasicBLAST" search using the "blastp" routine. See  
5 also Altschul et al. (1990) J Mol Biol 215:403-410. Preferably, such a composition does not result in an adverse immune response thereto when administered to a human recipient. In certain preferred embodiments, the subject antigen-binding domains of human composition include the framework regions of native human immunoglobulins, as may be cloned from activated human B cells, though not  
10 necessarily all of the CDRs of a native human antibody.

As used herein, the term "mini-antibody fragment" means a multivalent antibody fragment comprising at least two antigen-binding domains multimerized by self-associating domains fused to each of said domains (Pack, 1994), e.g. dimers  
15 comprising two scFv fragments, each fused to a self-associating dimerization domain. Dimerization domains, which are particularly preferred, include those derived from a leucine zipper (Pack and Plückthun, 1992) or helix-turn-helix motif (Pack et al., 1993).

As used herein, "activated cells" means cells of a certain population of interest, which  
20 are not resting. Activation might be caused by mitogens (e.g., lipopolysaccharide, phytohemagglutinine) or cytokines (e.g., interferon gamma). Preferably, said activation occurs during tumor transformation (e.g., by Epstein-Barr virus, or "spontaneously"). Preferably, activated cells are characterized by the features of MHC class II molecules expressed on the cell surface and one or more additional  
25 features including increased cell size, cell division, DNA replication, expression of CD45 or CD11 and production/secretion of immunoglobulin.

As used herein, "non-activated cells" means cells of a population of interest, which are resting and non-dividing. Said non-activated cells may include resting B cells as  
30 purified from healthy human blood. Such cells can, preferably, be characterized by lack or reduced level of MHC class II molecules expressed on the cell surface and lack or reduced level of one or more additional features including increased cell size, cell division, DNA replication, expression of CD45 or CD11 and production/secretion of immunoglobulin.

As used herein, the term "EC50" means the concentration of multivalent forms of the subject compositions which produces 50% of its maximum response or effect, such as cell killing.

5

"At least 5-fold lower EC50" means that the concentration of a multivalent composition comprising at least one polypeptide of the present invention that is required to kill 50% of activated cells is at least five times less than the concentration of the multivalent composition required to kill non-activated cells. Preferably, the concentration required to kill 50% of non-activated cells cannot be achieved with therapeutically appropriate concentrations of the multivalent composition. Most preferably, the EC50 value is determined in the test described below in the appended examples.

10

15 The term "immunosuppress" refers to the prevention or diminution of the immune response, as by irradiation or by administration of antimetabolites, antilymphocyte serum, or specific antibody.

The term "immune response" refers to any response of the immune system, or a cell forming part of the immune system (lymphocytes, granulocytes, macrophages, etc), to an antigenic stimulus, including, without limitation, antibody production, cell-mediated immunity, and immunological tolerance.

20

As used herein, the term "IC50" with respect immunosuppression, refers to the concentration of the subject compositions which produces 50% of its maximum response or effect, such as inhibition of an immune response, such as may be manifest by inhibition of IL2 secretion, down-regulation of IL2 expression, or reduced rate of cell proliferation.

25

30 The phrase "cytotoxic entities", with reference to a manner of cell killing, refers to mechanisms which are complement-dependent. Likewise, the phrase "immunological mechanism", with reference to a manner of cell killing, refers to macrophage-dependent and/or neutrophil-dependent killing of cells.

"Lymphoid cells" when used in reference to a cell line or a cell, means that the cell line or cell is derived from the lymphoid lineage. "Lymphoid cells" include cells of the B and the T lymphocyte lineages, and of the macrophage lineage.

- 5 Cells, which are "non lymphoid cells and express MHC class II", are cells other than lymphoid cells that express MHC class II molecules, e.g. during a pathological inflammatory response. For example, said cells may include synovial cells, endothelial cells, thyroid stromal cells and glial cells, and it may also comprise genetically altered cells capable of expressing MHC class II molecules.

10

The terms "apoptosis" and "apoptotic activity" refer to the form of cell death in mammals that is accompanied by one or more characteristic morphological and biochemical features, including nuclear and condensation of cytoplasm, chromatin aggregation, loss of plasma membrane microvilli, partition of cytoplasm and nucleus  
15 into membrane bound vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material, degradation of chromosomal DNA or loss of mitochondrial function. Apoptosis follows a very stringent time course and is executed by caspases, a specific group of proteases. Apoptotic activity can be determined and measured, for instance, by cell viability  
20 assays, Annexin V staining or caspase inhibition assays. Apoptosis can be induced using a cross-linking antibody such as anti-CD95 as described in Example H.

As used herein, the term "first domain of the  $\alpha$ -chain of HLA-DR" means the N-terminal domain of the alpha-chain of the MHC class II DR molecule.

25

As used herein, the term "first domain of the  $\beta$ -chain of HLA-DR" means the N-terminal domain of the beta-chain of the MHC class II DR molecule.

The term "innate pre-programmed process" refers to a process that, once it is started,  
30 follows an autonomous cascade of mechanisms within a cell, which does not require any further auxiliary support from the environment of said cell in order to complete the process.

As used herein, the term "HuCAL" refers to a fully synthetic human combinatorial antibody library as described in Knappik et al. (2000).

5 The term "variable region" as used herein in reference to immunoglobulin molecules has the ordinary meaning given to the term by the person of ordinary skill in the act of immunology. Both antibody heavy chains and antibody light chains may be divided into a "variable region" and a "constant region". The point of division between a variable region and a heavy region may readily be determined by the person of ordinary skill in the art by reference to standard texts describing antibody structure,  
10 e.g., Kabat et al "Sequences of Proteins of Immunological Interest: 5th Edition" U.S. Department of Health and Human Services, U.S. Government Printing Office (1991).

As used herein, the term "CDR3" refers to the third complementarity-determining region of the VH and VL domains of antibodies or fragments thereof, wherein the VH  
15 CDR3 covers positions 95 to 102 (possible insertions after positions 100 listed as 100a to 100z), and VL CDR3 positions 89 to 96 (possible insertions in VL after position 95 listed as 95a to 95c) (see Knappik et al., 2000).

As used herein, the term "hybridizes under stringent conditions" is intended to  
20 describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at least 65%, more preferably at least 70%, and even more preferably at least 75% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled  
25 in the art and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, New York. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions is hybridization in 6 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 x SSC, 0.1% SDS at 50°-65°C.

30 A "protein coding sequence" or a sequence which "encodes" a particular polypeptide or peptide, is a nucleic acid sequence which is transcribed (in the case of DNA) and translated (in the case of mRNA) into a polypeptide in vitro or in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a

translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but is not limited to, cDNA from procaryotic or eukaryotic mRNA, genomic DNA sequences from procaryotic or eukaryotic DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be located 3' to the coding  
5 sequence.

Likewise, "encodes", unless evident from its context, will be meant to include DNA sequences which encode a polypeptide, as the term is typically used, as well as DNA sequences which are transcribed into inhibitory antisense molecules.

10

As used herein, the term "transfection" means the introduction of a heterologous nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transient transfection" refers to cases where exogenous DNA does not integrate into the genome of a transfected cell, e.g., where episomal DNA is  
15 transcribed into mRNA and translated into protein. A cell has been "stably transfected" with a nucleic acid construct when the nucleic acid construct is capable of being inherited by daughter cells.

20

"Expression vector" refers to a replicable DNA construct used to express DNA which encodes the desired protein and which includes a transcriptional unit comprising an assembly of (1) agent(s) having a regulatory role in gene expression, for example, promoters, operators, or enhancers, operatively linked to (2) a DNA sequence encoding a desired protein (such as a polypeptide of the present invention) which is transcribed into mRNA and translated into protein, and (3) appropriate transcription  
25 and translation initiation and termination sequences. The choice of promoter and other regulatory elements generally varies according to the intended host cell. In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used  
30 form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

In the expression vectors, regulatory elements controlling transcription or translation can be generally derived from mammalian, microbial, viral or insect genes. The ability to replicate in a host, usually conferred by an origin of replication, and a selection gene to facilitate recognition of transformants may additionally be incorporated.

5 Vectors derived from viruses, such as retroviruses, adenoviruses, and the like, may be employed.

10 “Transcriptional regulatory sequence” is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters and the like which induce or control transcription of protein coding sequences with which they are operably linked. It will be understood that a recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of the gene, if any.

15 “Operably linked” when describing the relationship between two DNA regions simply means that they are functionally related to each other. For example, a promoter or other transcriptional regulatory sequence is operably linked to a coding sequence if it controls the transcription of the coding sequence.

20 As used herein, the term “fusion protein” is art recognized and refer to a chimeric protein which is at least initially expressed as single chain protein comprised of amino acid sequences derived from two or more different proteins, e.g., the fusion protein is a gene product of a fusion gene.

25 As used herein, “proliferating” and “proliferation” refer to cells undergoing mitosis.

The “growth rate” of a cell refers to the rate of proliferation of the cell and the state of differentiation of the cell.

30 The term “cell-proliferative disorder” denotes malignant as well as nonmalignant populations of transformed cells which morphologically often appear to differ from the surrounding tissue.

As used herein, "transformed cells" refers to cells which have spontaneously converted to a state of unrestrained growth, i.e., they have acquired the ability to grow through an indefinite number of divisions in culture. Transformed cells may be characterized by such terms as neoplastic, anaplastic and/or hyperplastic, with respect to their loss of growth control.

As used herein, "immortalized cells" refers to cells which have been altered via chemical and/or recombinant means such that the cells have the ability to grow through an indefinite number of divisions in culture.

As used herein the term "animal" refers to mammals, preferably mammals such as humans. Likewise, a "patient" or "subject" to be treated by the method of the invention can mean either a human or non-human animal.

According to the methods of the invention, the peptide may be administered in a pharmaceutically acceptable composition. In general, pharmaceutically-acceptable carriers for monoclonal antibodies, antibody fragments, and peptides are well-known to those of ordinary skill in the art. As used herein, the term "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. In preferred embodiments, the subject carrier medium which does not interfere with the effectiveness of the biological activity of the active ingredients and which is not excessively toxic to the hosts of the concentrations of which it is administered. The administration(s) may take place by any suitable technique, including subcutaneous and parenteral administration, preferably parenteral. Examples of parenteral administration include intravenous, intraarterial, intramuscular, and intraperitoneal, with intravenous being preferred.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or



dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required  
5 particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable  
10 compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active compounds, e.g., the subject polypeptides, in the required amount in the appropriate solvent with  
15 various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred  
20 methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

For oral administration the polypeptides of the present invention may be incorporated  
25 with excipients and used in the form of non-ingestible mouthwashes and dentifrices. A mouthwash may be prepared incorporating the active ingredient in the required amount in an appropriate solvent, such as a sodium borate solution (Dobell's Solution). The active ingredient may also be dispersed in dentifrices, including: gels, pastes, powders and slurries. The active ingredient may be added in a therapeutically  
30 effective amount to a paste dentifrice that may include water, binders, abrasives, flavoring agents, foaming agents, and humectants.

The compositions of the present invention may be formulated in a neutral or salt form. Pharmaceutically-acceptable salts include the acid addition salts (formed with the free

amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion, (see for example, "Remington's Pharmaceutical Sciences" 15th Edition, pages 1035-1038 and 1570-1580). Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

Upon formulation, solutions can be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms such as injectable solutions, drug release capsules and the like.

As used herein, the term "prophylactic or therapeutic" treatment refers to administration to the host of the medical condition. If it is administered prior to exposure to the condition, the treatment is prophylactic (i.e., it protects the host against tumor formation), whereas if administered after initiation of the disease, the treatment is therapeutic (i.e., it combats the existing tumor).

A multivalent composition of at least one polypeptide according to the invention is capable of causing cell death of activated cells, preferably lymphoid tumor cells without requiring any further additional measures such as chemotherapy and with limited immunogenic side effects on the treated patient. Further, the multivalent composition comprising a polypeptide according to the invention has the capability of binding to at least one epitope on the target antigen, however, several epitope binding sites might be combined in one molecule. Preferably, the multivalent composition comprising a polypeptide according to the invention shows at least 5-fold, or more preferably 10-fold higher killing activity against activated cells compared to non-activated cells. This higher activity on activated cells can be expressed as the at least 5-fold lower EC50 value on activated versus non-activated cells or as the higher percentage of killing of activated cells versus non-activated cells when using the same concentration of protein. Under the latter alternative, the multivalent composition comprising a polypeptide according to the invention at a given polypeptide concentration kills at least 50%, preferably at least 80%, of activated cells, whereas the same concentration of a multivalent composition comprising a polypeptide according to the invention under the same incubation conditions kills less than 15%, preferably less than 10% of the non-activated cells. The assay conditions for determining the EC50 value and the percentage killing activity are described below.

### **Brief Description of the Drawings**

#### ***Figure 1***

- a. Specificity of the anti-HLA-DR antibody fragments: Binding of MS-GPC-8-27-7, MS-GPC-8-27-10, MS-GPC-8-6-13, MS-GPC-8-27-41, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-6-27, MS-GPC-8 and MS-GPC-8-6 to HLA-DR protein, negative control proteins (BSA, testosterone-BSA, lysozyme and human apotransferrin), and an empty microtiter plate well (plastic). Specificity was assessed using standard ELISA procedures.
- b. Specificity of the anti-HLA-DR antibody fragments MS-GPC-1, 6, 8 & 10 isolated from the HuCAL library to HLA-DR protein, a mouse-human chimeric HLA protein and negative control proteins (lysozyme, transferrin, BSA and human  $\beta$ -globulin).

Specificity was assessed using standard ELISA procedures. A non-related antibody fragment (irr. scFv) was used as control.

**Figure 2**

5    Reactivity of the anti-HLA-DR antibody fragments (MS-GPC-1, 6, 8 and 10) and IgG forms of MS-GPC-8, MS-GPC-8-10-57, MS-GPC-8-27-41 & MS-GPC-8-6-17 to various cell lines expressing MHC class II molecules. "+" represents strong reactivity as detected using standard immunofluorescence procedure. "+/-" represents weak reactivity and "-" represents no detected reactivity between an anti-HLA-DR antibody  
10    fragment or IgG and a particular cell line.

**Figure 3**

Viability of tumor cells in the presence of monovalent and cross-linked anti-HLA-DR antibody fragments as assessed by trypan blue staining. Viability of GRANTA-519  
15    cells was assessed after 4 h incubation with anti-HLA-DR antibody fragments (MS-GPC-1, 6, 8 and 10) with and without anti-FLAG M2 mAb as cross-linking agent.

**Figure 4**

Scatter plots and fitted logistic curves of data from Table 5 showing improved killing efficiency of 50 nM solutions of the IgG form of the human antibody fragments of the invention treated compared to treatment with 200 nM solutions of murine antibodies. Open circles represent data for cell lines treated with the murine antibodies L243 and 8D1 and closed circles for human antibodies MS-GPC-8, MS-GPC-8-27-41, MS-GPC-8-10-57 and MS-GPC-8-6-13. Fitted logistic curves for human (solid) and  
20    mouse (dashed) mAb cell killing data show the overall superiority of the treatment with human mAbs at 50 nM compared to the mouse mAbs despite treatment at a final concentration of 200 nM.  
25

30    **Figure 5**

Killing of activated versus non-activated cells. MHH-PREB-1 cells are activated with Lipopolysaccharide, Interferon-gamma and phyto-hemagglutinin, and subsequently incubated for 4 h with 0.07 to 3300 nM of the IgG forms of the anti-HLA-DR antibody

fragments MS-GPC-8-10-57 and MS-GPC-8-27-41. No loss of viability in the control non-activated MHH-PREB-1 cells is seen.

**Figure 6**

- 5 Killing efficiency of control (no antibody, unreactive murine IgG; light grey), and human (MS-GPC-8, MS-GPC-8-10-57 & MS-GPC-8-27-41; dark grey) IgG forms of anti-HLA-DR antibody fragments against CLL cells isolated from patients. Left panel, box-plot display of viability data from 10 patient resting cell cultures against antibodies after incubation for four (h4) and twenty four hours (h24). Right panel box-plot display of viability data from 6 patient activated cell cultures against antibodies after incubation for four (h4) and twenty four hours (h24).

**Figure 7**

- 15 Concentration dependent cell viability for certain anti-HLA-DR antibody fragments of the invention. Vertical lines indicate the EC50 value estimated by logistic non-linear regression on replica data obtained for each of the antibody fragments. a) Killing curves of cross-linked bivalent anti-HLA-DR antibody F(ab) fragment dimers MS-GPC-10 (circles and solid line), MS-GPC-8 (triangles and dashed line) and MS-GPC-1 (crosses and dotted line). b) Killing curves of cross-linked bivalent anti-HLA-DR antibody (Fab) fragment dimers MS-GPC-8-17 (circles and solid line), and murine IgGs 8D1 (triangles and dashed line) and L243 (crosses and dotted line). c) Killing curves of cross-linked bivalent anti-HLA-DR antibody (Fab) fragment dimers GPC-8-6-2 (crostriangles and dashed line), and murine IgGs 8D1 (circles and solid line) and L243 (crosses and dotted line). d) Killing curves of IgG forms of human anti-HLA-DR antibody fragments MS-GPC-8-10-57 (crosses and dotted line), MS-GPC-8-27-41 (exes and dash-dot line), and murine IgGs 8D1 (circles and solid line) and L243 (triangles and dashed line). All concentrations are given in nM of the bivalent agent (IgG or cross-linked (Fab) dimer).

**Figure 8**

- a. Incubation of Priess cells with the anti-HLA-DR antibody fragment MS-GPC-8, cross-linked using the anti-FLAG M2 mAb, shows more rapid killing than a culture of

Priess cells induced into apoptosis using anti-CD95 mAb. An Annexin V/PI staining technique identifies necrotic cells by Annexin V positive and PI positive staining.

- 5 b. Incubation of Priess cells with the anti-HLA-DR antibody fragment MS-GPC-8, cross-linked using the anti-FLAG M2 mAb, shows little evidence of an apoptotic mechanism compared to an apoptotic culture of Priess cells induced using anti-CD95 mAb. An Annexin V/PI staining technique identifies apoptotic cells by Annexin V positive and PI negative staining.

10 **Figure 9**

a. Immunosuppressive properties of the IgG forms of the anti-HLA-DR antibody fragments MS-GPC-8-10-57, MS-GPC-8-27-41 & MS-GPC-8-6-13 using an assay to determine inhibition of IL-2 secretion from T-hybridoma cells.

- 15 b. Immunosuppressive properties of the monovalent Fab forms of the anti-HLA-DR antibody fragments MS-GPC-8-27-41 & MS-GPC-8-6-19 using an assay to determine inhibition of IL-2 secretion from T-hybridoma cells

**Figure 10**

- 20 Immunosuppressive properties of the IgG forms of the anti-HLA-DR antibody fragments MS-GPC-8-10-57 and MS-GPC-8-27-41 in an assay to determine inhibition of T cell proliferation.

**Figure 11**

Vector map and sequence of scFv phage display vector pMORPH13\_scFv.

- 25 The vector pMORPH13\_scFv is a phagemid vector comprising a gene encoding a fusion between the C-terminal domain of the gene III protein of filamentous phage and a HuCAL scFv. In Figure 11, a vector comprising a model scFv gene (combination of VH1A and V $\lambda$ 3 (Knappik et al., 2000) is shown.

- 30 The original HuCAL master genes (Knappik et al. (2000): see Fig. 3 therein) have been constructed with their authentic N-termini: VH1A, VH1B, VH2, VH4 and VH6 with Q (=CAG) as the first amino acid. VH3 and VH5 with E (=GAA) as the first amino acid. Vector pMORPH13\_scFv comprises the short FLAG peptide sequence (DYKD) fused to the VH chain, and thus all HuCAL VH chains in, and directly derived from,

this vector have E (=GAA) at the first position (e.g. in pMx7\_FS vector, see Figure 12).

### **Figure 12**

5 Vector map and sequence of scFv expression vector pMx7\_FS\_5D2.

The expression vector pMx7\_FS\_5D2 leads to the expression of HuCAL scFv fragments (in Figure 12, the vector comprises a gene encoding a "dummy" antibody fragment called "5D2") when VH-CH1 is fused to a combination of a FLAG tag (Hopp et al., 1988; Knappik and Plückthun, 1994) and a STREP tag II (WSHPQFEK) (IBA  
10 GmbH, Göttingen, Germany; see: Schmidt and Skerra, 1993; Schmidt and Skerra, 1994; Schmidt et al., 1996; Voss and Skerra, 1997).

### **Figure 13**

Vector map and sequence of Fab expression vector pMx9\_Fab\_GPC8.

15 The expression vector pMx9\_Fab\_GPC8 leads to the expression of HuCAL Fab fragments (in Figure 13, the vector comprises the Fab fragment MS-GPC8) when VH-CH1 is fused to a combination of a FLAG tag (Hopp et al., 1988; Knappik and Plückthun, 1994) and a STREP tag II (WSHPQFEK) (IBA GmbH, Göttingen, Germany; see: Schmidt and Skerra, 1993; Schmidt and Skerra, 1994; Schmidt et al.,  
20 1996; Voss and Skerra, 1997).

In pMx9\_Fab vectors, the HuCAL Fab fragments cloned from the scFv fragments (see figure caption of Figure 11) do not have the short FLAG peptide sequence (DYKD) fused to the VH chain, and all HuCAL VH chains in, and directly derived from, that vector have Q (=CAG) at the first position

25

### **Figure 14**

Vector map and sequence of Fab phage display vector pMORPH18\_Fab\_GPC8.

The derivatives of vector pMORPH18 are phagemid vectors comprising a gene encoding a fusion between the C-terminal domain of the gene III protein of filamentous phage and the VH-CH1 chain of a HuCAL antibody. Additionally, the  
30 vector comprises the separately encoded VL-CL chain. In Figure 14, a vector comprising the Fab fragment MS-GPC-8 is shown.

In pMORPH18\_Fab vectors, the HuCAL Fab fragments cloned from the scFv fragments (see figure caption of Figure 11) do not have the short FLAG peptide

sequence (DYKD) fused to the VH chain, and all HuCAL VH chains in, and directly derived from, that vector have Q (=CAG) at the first position.

### **Figure 15**

5 Amino acid sequences of VH and VL domains of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-6, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-27, MS-GPC-8-6-13, MS-GPC-8-10-57, and MS-GPC-8-27-41.

The sequences in Figure 15 show amino acid 1 of VH as constructed in the original HuCAL master genes (Knappik et al. (2000): see Fig. 3 therein). In scFv constructs,  
10 as described in this application, amino acid 1 of VH is always E (see figure caption of Figure 11), in Fab constructs as described in this application, amino acid 1 of VH is always Q (see figure caption of Figure 13)

### **Detailed Description of the Invention**

15 The following examples illustrate the invention.

### ***Examples***

All buffers, solutions or procedures without explicit reference can be found in standard textbooks, for example Current Protocols of Immunology (1997 and 1999) or  
20 Sambrook et al., 1989. Where not given otherwise, all materials were purchased from Sigma, Deisenhofen, DE, or Merck, Darmstadt, DE, or sources are given in the literature cited. Hybridoma cell lines LB3.1 and L243 were obtained from LGC Reference Materials, Middlesex, UK; data on antibody 8D1 were generously supplied by Dr. Matyas Sandor, University of Michigan, Madison, WI, USA.

25

#### ***1. Preparation of a human antigen***

To demonstrate that we could identify cytotoxic antigen-binding domains of human composition, we first prepared a purified form of a human antigen, the human MHC class II DR protein (DRA\*0101/DRB1\*0401) from PRIESS cells (Gorga et al., 1984;  
30 Gorga et al., 1986; Gorga et al., 1987; Stern et al., 1992) as follows.

First, PRIESS cells (ECACC, Salisbury UK) were cultured in RPMI and 10% fetal calf serum (FCS) using standard conditions, and  $10^{10}$  cells were lysed in 200 ml phosphate buffered saline (PBS) (pH 7.5) containing 1% NP-40 (BDH, Poole, UK), 25



mM iodoacetamide, 1 mM phenylmethylsulfonylfluoride (PMSF) and 10 mg/l each of the protease inhibitors chymostatin, antipain, pepstatin A, soybean trypsin inhibitor and leupeptin. The lysate was centrifuged at 10.000 g (30 minutes, 4°C) and the resulting supernatant was supplemented with 40 ml of an aqueous solution containing 5% sodium deoxycholate, 5 mM iodoacetamide and 10 mg/l each of the above protease inhibitors and centrifuged at 100.000 g for two hours (4°C). To remove material that bound non-specifically and endogenous antibodies, the resulting supernatant was made 0.2 mM with PMSF and passed overnight (4°C) through a rabbit serum affigel-10 column (5 ml; for preparation, rabbit serum (Charles River, Wilmington, MA, USA) was incubated with Affigel 10 (BioRad, Munich, DE) at a volume ratio of 3:1 and washed following manufacturer's directions) followed by a Protein G Sepharose Fast Flow column (2 ml; Pharmacia) using a flow rate of 0.2 ml/min.

Second, the pre-treated lysate was batch incubated with 5 ml Protein G Sepharose Fast Flow beads coupled to the murine anti-HLA-DR antibody LB3.1 (obtained by Protein G-Sepharose FF (Pharmacia) affinity chromatography of a supernatant of hybridoma cell line LB3.1) (Stern et al., 1993) overnight at 4°C using gentle mixing, and then transferred into a small column which was then washed extensively with three solutions: (1) 100 ml of a solution consisting of 50 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 10% glycerol and 0.03% sodium azide at a flow rate of 0.6 ml/min). (2) 25 ml of a solution consisting of 50 mM Tris/HCl (pH 9.0), 0.5 M NaCl, 0.5 % NP-40, 0.5% sodium deoxycholate, 10% glycerol and 0.03% sodium azide at a flow rate of 0.9 ml/min; (3) 25 ml of a solution consisting of 2 mM Tris/HCl (pH 8.0), 1% octyl-β-D-glucopyranoside, 10% glycerol and 0.03% sodium azide at a flow rate of 0.9 ml/min.

Third, MHC class II DR protein (DRA\*0101/DRB1\*0401) was eluted using 15 ml of a solution consisting of 50 mM diethylamine/HCl (pH 11.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% octyl-β-D-glucopyranoside (Alexis Corp., Lausen, CH), 10% glycerol, 10 mM iodoacetamide and 0.03% sodium azide at a flow rate of 0.4 ml/min. 800 µl fractions were immediately neutralised with 100 µl 1M Tris/HCl (pH 6.8), 150 mM NaCl and 1% octyl-β-D-glucopyranoside. The incubation of the lysate with LB3.1-Protein G Sepharose Fast Flow beads was repeated until the lysate was exhausted of

MHC protein. Pure eluted fractions of the MHC class II DR protein (as analyzed by SDS-PAGE) were pooled and concentrated to 1.0-1.3 g/l using Vivaspin concentrators (Greiner, Solingen, DE) with a 30 kDa molecular weight cut-off. Approximately 1 mg of the MHC class II DR preparation was re-buffered with PBS  
5 containing 1% octyl- $\beta$ -D-glucopyranoside using the same Vivaspin concentrator to enable direct coupling of the protein to BIAcore CM5 chips.

## 2. Screening of HuCAL

### 2.1. Introduction

10 We identified certain antigen binding antibody fragments of human composition (MS-GPC-1, MS-GP-6, MS-GPC-8 and MS-GPC-10) against the human antigen (DRA\*0101/DRB1\*0401) from a human antibody library based on a novel concept that has been recently developed (Knappik et al., 2000). A consensus framework resulting in a total of 49 different frameworks here represents each of the VH- and  
15 VL-subfamilies frequently used in human immune responses. These master genes were designed to take into account and eliminate unfavorable residues promoting protein aggregation as well as to create unique restriction sites leading to modular composition of the genes. In HuCAL-scFv, both the VH- and VL-CDR3 encoding regions of the 49 master genes were randomized.

20

### 2.2. Phagemid rescue, phage amplification and purification

The HuCAL-scFv (Knappik et al., 2000) library, cloned into a phagemid-based phage display vector pMORPH13\_scFv (see Figure 11), in *E.coli* TG-1 was amplified in 2 x TY medium containing 34  $\mu$ g/ml chloramphenicol and 1% glucose (2 x TY-CG). After  
25 helper phage infection (VCSM13) at 37°C at an OD<sub>600</sub> of about 0.5, centrifugation and resuspension in 2 x TY / 34  $\mu$ g/ml chloramphenicol / 50  $\mu$ g/ml kanamycin / 0.1 mM IPTG, cells were grown overnight at 30°C. Phage were PEG-precipitated from the supernatant (Ausubel et al., 1998), resuspended in PBS/20% glycerol and stored at –80°C. Phage amplification between two panning rounds was conducted as follows:  
30 mid-log phase TG1-cells were infected with eluted phage and plated onto LB-agar supplemented with 1% of glucose and 34  $\mu$ g/ml of chloramphenicol. After overnight incubation at 30°C colonies were scraped off, adjusted to an OD<sub>600</sub> of 0.5 and helper phage added as described above.

### 2.3. Manual solid phase panning

Wells of MaxiSorp™ microtiterplates (Nunc, Roskilde, DK) were coated with MHC-class II DRA\*0101/DRB1\*0401 (prepared as above) dissolved in PBS (2 µg/well). After blocking with 5% non-fat dried milk in PBS,  $1-5 \times 10^{12}$  HuCAL-scFv phage purified as above were added for 1h at 20°C. After several washing steps, bound phages were eluted by pH-elution with 100 mM triethylamine and subsequent neutralization with 1M TRIS-Cl pH 7.0. Three rounds of panning were performed with phage amplification conducted between each round as described above.

### 2.4. Mixed solid phase/whole cell panning

Three rounds of panning and phage amplification were performed as described in 2.3. and 2.2. with the exception that in the second round between  $1 \times 10^7$  and  $5 \times 10^7$  PRIESS cells in 1 ml PBS/10% FCS were used in 10 ml Falcon tubes for whole cell panning. After incubation for 1h at 20°C with the phage preparation, the cell suspension was centrifuged (2000 rpm for 3 min) to remove non-binding phage, the cells were washed three times with 10 ml PBS, each time followed by centrifugation as described. Phage that specifically bound to the cells were eluted off by pH-elution using 100 mM HCl. Alternatively, binding phage could be amplified by directly adding E.coli to the suspension after triethylamine treatment (100 mM) and subsequent neutralization.

### 2.5 Identification of HLA-DR binding scFv fragments

Clones obtained after three rounds of solid phase panning (2.3) or mixed solid phase/whole cell panning (2.4) were screened by FACS analysis on PRIESS cells for binding to HLA-DR on the cell surface. For expression, the scFv fragments were cloned via XbaI/EcoRI into pMx7\_FS as expression vector (see Figure 12). Expression conditions are shown below in example 3.2

Aliquots of  $10^6$  Priess cells were transferred at 4°C into wells of a 96-well microtiterplate. ScFv in blocking buffer (PBS/5% FCS) were added for 60 min and detected using an anti-FLAG M2 antibody (Kodak) (1:5000 dilution) followed by a polyclonal goat anti-mouse IgG antibody-R-Phycoerythrin-conjugate (Jackson ImmunoResearch, West Grove, PA, USA, Cat. No. 115-116-146, F(ab')<sub>2</sub> fragment) (1:200 dilution). Cells were fixed in 4% paraformaldehyde for storage at 4°C.  $10^4$

events were collected for each assay on the FACS-Calibur (BD Immunocytometry Systems, San Jose, CA, USA).

Only fifteen out of over 500 putative binders were identified which specifically bound to Priess cells. These clones were further analyzed for their killing activity as described below. Table 1 contains the sequence characteristics of clones MS-GPC-1, MS-GPC-6, MS-GPC-8 and MS-GPC-10 identified thereby. The VH and VL families and the CDR3s listed refer to the HuCAL consensus-based antibody genes as described (Knappik et al., 2000); the sequences of the VH and VL CDRs are shown in Table 1, and the full sequences of the VH and VL domains are shown in Figure 15.

### 3. Generation of Fab-fragments

#### 3.1. Conversion of scFv to Fab

The Fab-fragment antigen binding polypeptides MS-GPC-1-Fab, MS-GP-6-Fab, MS-GPC-8-Fab and MS-GPC-10-Fab were generated from their corresponding scFv fragments as follows. Both heavy and light chain variable domains of scFv fragments were cloned into pMx9\_Fab (Figure 13), the heavy chain variable domains as MfeI / StyI-fragments, the variable domains of the kappa light chains as EcoRV/ BsiWI-fragments. The lambda chains were first amplified from the corresponding pMORPH13\_scFv vector as template with PCR-primers CRT5 (5' primer) and CRT6 (3' primer), wherein CRT6 introduces a unique DraIII restriction endonuclease site.

CRT5: 5' GTGGTGGTTCCGATATC 3'

CRT6: 5' AGCGTCACACTCGGTGCGGCTTTCGGCTGGCCAAGAACGGGTTA 3'

The PCR product is cut with EcoRV / DraIII and cloned into pMx9\_Fab (see Figure 13). The Fab light chains could be detected with a polyclonal goat anti-human IgG antibody-R-Phycoerythrin-conjugate (Jackson ImmunoResearch, West Grove, PA, USA, Cat. No. 109-116-088, F(ab')<sub>2</sub> fragment) (1:200 dilution).

#### 3.2. Expression and purification of HuCAL-antibody fragments in E.coli

Expression in E.coli cells (JM83) of scFv and Fab fragments from pMx7\_FS or pMx9\_Fab, respectively, were carried out in one litre of 2 x TY-medium supplemented

with 34 µg/ml chloramphenicol. After induction with 0.5 mM IPTG (scFv) or 0.1 mM IPTG (Fab), cells were grown at 22°C for 12 hours. Cell pellets were lysed in a French Press (Thermo Spectronic, Rochester, NY, USA) in 20 mM sodium phosphate, 0.5 M NaCl, and 10 mM imidazole (pH 7.4). Cell debris was removed by centrifugation and the clear supernatant filtered through 0.2 µm pores before subjecting it to STREP tag purification using a Streptactin matrix and purification conditions according to the supplier (IBA GmbH, Göttingen, Germany). Purification by size exclusion chromatography (SEC) was performed as described by Rheinacker et al. (1996). The apparent molecular weights were determined by SEC with calibration standards and confirmed in some instances by coupled liquid chromatography-mass spectrometry (TopLab GmbH, Martinsried, Germany).

#### *4. Optimization of antibody fragments*

In order to optimize certain biological characteristics of the HLA-DR binding antibody fragments, one of the Fab fragments, MS-GPC-8-Fab, was used to construct a library of Fab antibody fragments by replacing the parental VL λ1 chain by the pool of all lambda chains λ 1-3 randomized in CDR3 from the HuCAL library (Knappik et al., 2000).

The Fab fragment MS-GPC-8-Fab (see 3.1) was cloned via XbaI/EcoRI from pMx9\_Fab\_GPC-8 into pMORPH18\_Fab, a phagemid-based vector for phage display of Fab fragments, to generate pMORPH18\_Fab\_GPC-8 (see Figure 14). A lambda chain pool comprising a unique DraIII restriction endonuclease site (Knappik et al., 2000) was cloned into pMORPH18\_Fab\_GPC-8 cut with NsiI and DraIII (see vector map of pMORPH18\_Fab\_GPC-8 in Figure 14).

The resulting Fab optimization library was screened by two rounds of panning against MHC-class II DRA\*0101/DRB1\*0401 (prepared as above) as described in 2.3 with the exception that in the second round the antigen concentration for coating was decreased to 12 ng/well. FACS identified optimized clones as described above in 2.5. Seven of these clones, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18 and MS-GPC-8-27, were further characterized and showed cell killing activity as found for the starting fragment MS-GPC-8. Table 1 contains the sequence characteristics of MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9,

MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18 and MS-GPC-8-27. The VH and VL families and the CDR3s listed refer to the HuCAL consensus-based antibody genes as described (Knappik et al., 2000). The full sequences of the VH and VL domains of MS-GPC-8-6, MS-GPC-8-10, MS-GPC-8-17 and MS-GPC-8-27 are shown in Figure 15.

The optimized Fab forms of the anti-HLA-DR antibody fragments MS-GPC-8-6 and MS-GPC-8-17 showed improved characteristics over the starting MS-GPC-8. For example, the EC<sub>50</sub> of the optimized antibodies was 15-20 and 5-20 nM (compared to 20-40 nM for MS-GPC-8, where the concentration is given as the concentration of the bivalent cross-linked Fab dimer), and the maximum capacity to kill MHH-Cell 4 cells determined as 76 and 78% for MS-GPC-8-6 and MS-GPC-8-17 (compared to 65% for MS-GPC-8) respectively.

For further optimization, the VL CDR1 regions of a set of anti-HLA-DR antibody fragments derived from MS-GPC-8 (including MS-GPC-8-10 and MS-GPC-8-27) were optimized by cassette mutagenesis using trinucleotide-directed mutagenesis (Virnekäs et al., 1994). In brief, a VL CDR1 library cassette was synthesized containing six randomized positions (total variability:  $7.43 \times 10^6$ ), and was cloned into a VL framework. The CDR1 library was digested with EcoRV and BbsI, and the fragment comprising the CDR1 library ligated into the lambda light chains of the MS-GPC-8-derived Fab antibody fragments in pMORPH18\_Fab (as described above), digested with EcoRV and BbsI. The resulting library was screened as described above. Ten clones were identified as above by binding specifically to HLA DR (MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 & MS-GPC-8-27-41) and showed cell killing activity as found for the starting fragments MS-GPC-8, MS-GPC-8-10 and MS-GPC-8-27. Table 1 contains the sequence characteristics of MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 & MS-GPC-8-27-41. The VH and VL families and the CDR3s listed refer to the HuCAL consensus-based antibody genes as described (Knappik et al., 2000), the full sequences of the VH and VL domains of MS-GPC-8-6-13, MS-GPC-8-10-57 & MS-GPC-8-27-41 are shown in Figure 15.

From these 10 clones, four Fab fragments were chosen (MS-GPC-8-6-2, MS-GPC-8-6-13, MS-GPC-8-10-57 and MS-GPC-8-27-41) as demonstrating significantly improved EC50 of cell killing as described in example 10. Table 1 shows the sequences of clones optimised at the CDR1 region.

Optimisation procedures not only increased the biological efficacy of anti-HLA DR antibody fragments generated by the optimisation process, but a physical characteristic - affinity of the antibody fragment to HLA DR protein - was also substantially improved. For example, the affinity of Fab forms of MS-GPC-8 and its optimised descendents was measured using a surface plasmon resonance instrument (Biacore, Upsala Sweden) according to example 7. The affinity of the MS-GPC-8 parental Fab was improved over 100 fold from 346 nM to ~ 60 nM after VLCDR3 optimisation and further improved to single digit nanomolar affinity (range 3 – 9 nM) after VLCDR3+1 optimisation (Table 2).

## 5. Generation of IgG

### 5.1 Construction of HuCAL-immunoglobulin expression vectors

Heavy chains were cloned as follows. The multiple cloning site of pcDNA3.1+ (Invitrogen) was removed (NheI / ApaI), and a stuffer compatible with the restriction sites used for HuCAL-design was inserted for the ligation of the leader sequences (NheI / EcoRI), VH-domains (EcoRI / BlnI) and the immunoglobulin constant regions (BlnI / ApaI). The leader sequence (EMBL M83133) was equipped with a Kozak sequence (Kozak, 1987). The constant regions of human IgG1 (PIR J00228), IgG4 (EMBL K01316) and serum IgA1 (EMBL J00220) were dissected into overlapping oligonucleotides with lengths of about 70 bases. Silent mutations were introduced to remove restriction sites non-compatible with the HuCAL-design. The oligonucleotides were spliced by overlap extension-PCR.

Light chains were cloned as follows. The multiple cloning site of pcDNA3.1/Zeo+ (Invitrogen) was replaced by two different stuffers. The  $\kappa$ -stuffer provided restriction sites for insertion of a  $\kappa$ -leader (NheI / EcoRV), HuCAL-scFv V $\kappa$ -domains (EcoRV / BsiWI) and the  $\kappa$ -chain constant region (BsiWI / ApaI). The corresponding restriction

5 sites in the  $\lambda$ -stuffer were NheI / EcoRV ( $\lambda$ -leader), EcoRV / HpaI ( $V\lambda$ - domains) and HpaI / ApaI ( $\lambda$ -chain constant region). The  $\kappa$ -leader (EMBL Z00022) as well as the  $\lambda$ -leader (EMBL L27692) were both equipped with Kozak sequences. The constant regions of the human  $\kappa$ - (EMBL J00241) and  $\lambda$  -chain (EMBL M18645) were assembled by overlap extension-PCR as described above.

## 5.2 Generation of IgG-expressing CHO-cells

10 All cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in media recommended by the supplier. CHO-K1 (CRL-9618) were from ATCC and were co-transfected with an equimolar mixture of IgG heavy and light chain expression vectors. Double-resistant transfectants were selected with 600  $\mu$ g/ml G418 and 300  $\mu$ g/ml Zeocin (Invitrogen) followed by limiting dilution. The supernatant of single clones was assessed for IgG expression by capture-ELISA. Positive clones were expanded in RPMI-1640 medium supplemented with 10% ultra-low IgG-FCS (Life  
15 Technologies). After adjusting the pH of the supernatant to 8.0 and sterile filtration, the solution was subjected to standard protein A column chromatography (Poros 20A, PE Biosystems).

20 The IgG forms of anti-HLA-DR antigen binding domains show improved characteristics over the antibody fragments. These improved characteristics include affinity (Example 7) and killing efficiency (Examples 9, 10 and 14).

## 6. HLA-DR specificity assay and epitope mapping

25 To demonstrate that antigen-binding domains selected from the HuCAL library bound specifically to a binding site on the N-terminal domain of human MHCII receptor largely conserved between alleles and hitherto unknown in the context of cell killing by receptor cross linking, we undertook an assessment of their binding specificity, and it was attempted to characterise the binding epitope.

30 The Fab antibody fragments MS-GPC-8-27-7, MS-GPC-8-27-10, MS-GPC-8-6-13, MS-GPC-8-27-41, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-6-27, MS-GPC-8 and MS-GPC-8-6 showed specificity of binding to HLA-DR protein but not to non-HLA-DR proteins. Fab fragments selected from the HuCAL library were tested for reactivity with the following antigens: HLA-DR protein (DRA\*0101/DRB1\*0401;



prepared as example 1, and a set of unrelated non-HLA-DR proteins consisting of BSA, testosterone-BSA, lysozyme and human apotransferrin. An empty well (Plastic) was used as negative control. Coating of the antigen MHCII was performed over night at 1 µg/well in PBS (Nunc-MaxiSorp TM) whereas for the other antigens (BSA, Testosterone-BSA, Lysozyme, Apotransferrin) 10 µg/well was used. Next day wells were blocked in 5% non-fat milk for 1 hr followed by incubation of the respective antibodies (anti-MHCII-Fabs and an unrelated Fab (Mac1-8A)) at 100 ng/well for 1h. After washing in PBS the anti-human IgG F(ab')<sub>2</sub>-peroxidase-conjugate at a 1:10000 dilution in TBS (supplemented with 5% w/v non-fat dry-milk/0.05% v/v Tween 20) was added to each well for 1h. Final washes were carried out in PBS followed the addition the substrate POD (Roche). Color-development was read at 370 nM in an ELISA-Reader

All anti-HLA-DR antibody fragments MS-GPC-8-27-7, MS-GPC-8-27-10, MS-GPC-8-6-13, MS-GPC-8-27-41, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-6-27, MS-GPC-8 and MS-GPC-8-6 demonstrated high specificity for HLA-DR, as evidenced by the much higher mean fluorescence intensity resulting from incubation of these antibody fragments with HLA-DR derived antigens compared to controls (Figure 1a). In a similar experiment, the Fab fragments MS-GPC-1, MS-GPC-6, MS-GPC-8 and MS-GPC-10 were found to bind to both the DRA\*0101/DRB1\*0401 (prepared as above) as well as to a chimeric DR-IE consisting of the N-terminal domains of DRA\*0101 and DRB1\*0401 with the remaining molecule derived from a murine class II homologue IEd (Ito et al., 1996) (Figure 1b).

To demonstrate the broad-DR reactivity of anti-HLA-DR antibody fragments and IgGs of the invention, the scFv forms of MS-GPC-1, 6, 8 and 10, and IgG forms of MS-GPC-8, MS-GPC-8-10-57, MS-GPC-8-27-51 & MS-GPC-8-6-13 were tested for reactivity against a panel of Epstein-Barr virus transformed B cell lines obtained from ECACC (Salisbury UK), each homozygous for one of the most frequent DR alleles in human populations (list of cell lines and alleles shown in Figure 2). The antibody fragments were also tested for reactivity against a series of L cells transfected to express human class II isotypes other than DRB1: L105.1, L257.6, L25.4, L256.12 & L21.3 that express the molecules DRB3\*0101, DRB4\*0101, DP0103/0402, DP0202/0201, and DQ0201/0602 respectively (Klohe et al., 1988).

Reactivity of an antigen-binding fragment to the panel of cell-lines expressing various MHC- class II molecules was demonstrated using an immunofluorescence procedure as for example, described by Otten et al (1997). Staining was performed on  $2 \times 10^5$  cells using an anti-FLAG M2 antibody as the second reagent against the M2 tag carried by each anti-HLA-DR antibody fragment and a fluorescein labelled goat anti-mouse Ig (BD Pharmingen, Torrey Pine, CA, USA) as a staining reagent. Cells were incubated at  $4^\circ\text{C}$  for 60 min with a concentration of 200 nM of the anti-HLA-DR antibody fragment, followed by the second and third antibody at concentrations determined by the manufacturers. For the IgG form, the second antibody was omitted and the IgG detected using a FITC-labeled mouse anti-human IgG4 (Serotec, Oxford, UK) . Cells were washed between incubation steps. Finally the cells were washed and subjected to analysis using a FACS Calibur (BD Immunocytometry Systems, San Jose, CA, USA).

Figure 2 shows that the scFv-fragments MS-GPC-1, 6, 8 and 10, and IgG forms of MS-GPC-8, MS-GPC-8-10-57, MS-GPC-8-27-51 & MS-GPC-8-6-13 react with all DRB1 allotypes tested. This observation taken together with the observation that all anti-HLA-DR antibody fragments react with chimeric DR-IE, suggests that all selected anti-HLA-DR antibody fragments recognize the extracellular first domain of the monomorphic DR $\alpha$  chain or a monomorphic epitope on extracellular first domain of the DR $\beta$  chain.

We then attempted to localize the binding domains of MS-GPC-8-10-57 and MS-GPC-8-27-41 further by examining competitive binding with murine antibodies for which the binding domains on HLA-DR are known. The murine antibodies L243 and LB3.1 are known to bind to the  $\alpha 1$  domain, 1-1C4 and 8D1 to the  $\beta 1$  domain and 10F12 to the  $\beta 2$  domain (Vidovic et al. 1995b). To this end, an assay was developed wherein a DR-expressing cell line (LG-2) was at first incubated with the IgG4 forms of MS-GPC-8-10-57 or MS-GPC-8-27-41, the Fab form of MS-GPC-8-10-57 or the Fab form of GPC 8, and an unrelated control antibody. Subsequently murine antibodies were added and the murine antibodies were detected. If the binding site of MS-GPC-8-10-57 or MS-GPC-8-27-41 overlaps with the binding of a murine antibody, then a reduced detection of the murine antibody is expected.

Binding of the IgG4 forms of GPC-8-27-41 and MS-GPC-8-10-57 and the Fab form of MS-GPC-8-10-57 substantially inhibited (mean fluorescence intensity reduced by > 90%) the binding of 1-1C4 and 8D1, whereas L243, LB3.1 and 10F12 and a control were only marginally affected. The Fab form of MS-GPC-8 reduced binding of 1-1C4 by ~ 50% (mean fluorescence dropped from 244 to 118), abolished 8D1 binding and only marginally affected binding of L243, LB3.1 and 10F12 or the control. An unrelated control antibody had no effect on either binding. Thus, MS-GPC-8-10-57 and MS-GPC-8-27-41 seem to recognise a  $\beta$ 1 domain epitope that is highly conserved among allelic HLA-DR molecules.

The whole staining procedure was performed on ice.  $1 \times 10^7$  cells of the human B-lymphoblastoid cell line LG-2 was preblocked for 20 Min. in PBS containing 2% FCS and 35  $\mu$ g/ml Guinea Pig IgG ("FACS-Buffer"). These cells were divided into 3 equal parts A, B, and C of approximately  $3.3 \times 10^6$  cells each, and it was added to A.) 35  $\mu$ g MS-GPC-8-10-57 or MS-GPC-8-27-41 IgG4, to B.) 35  $\mu$ g MS-GPC-8-10-57 Fab or MS-GPC-8 Fab, and to C.) 35  $\mu$ g of an unrelated IgG4 antibody as negative control, respectively, and incubated for 90 min. Subsequently A, B, C were divided in 6 equal parts each containing  $5.5 \times 10^5$  cells, and 2  $\mu$ g of the following murine antibodies were added each to one vial and incubated for 30 min: 1.) purified mIgG ; 2.) L243; 3.) LB3.1; 4.) 1-1 C4; 5.) 8D1; 6.) 10F12. Subsequently, 4ml of PBS were added to each vial, the vials were centrifuged at 300g for 8 min, and the cell pellet resuspended in 50  $\mu$ l FACS buffer containing a 1 to 25 dilution of a goat-anti-murine Ig-FITC conjugate at 20  $\mu$ g/ml final concentration (BD Pharmingen, Torrey Pines, CA, USA). Cells were incubated light-protected for 30 min. Afterwards, cells were washed with 4 ml PBS, centrifuged as above and resuspended in 500  $\mu$ l PBS for analysis in the flow cytometer (FACS Calibur, BD Immunocytometry Systems, San Jose, CA, USA).

The PepSpot technique (US 6040423; Heiskanen et al., 1999) is used to further identify the binding epitope for MS-GPC 8-10-57. Briefly, an array of 73 overlapping 15mer peptides is synthesised on a cellulose membrane by a solid phase peptide synthesis spotting method (WO 00/12575). These peptide sequences are derived from the sequence of the  $\alpha$ 1 and  $\beta$ 1 domains of HLA-DR4Dw14, HLA-DRA1\*0101 (residues 1-81) and HLA-DRB1\*0401 (residues 2-92), respectively, and overlap by

two amino acids. Second, such an array is soaked in 0.1% Tween-20/PBS (PBS-T), blocked with 5% BSA in PBS-T for 3 hours at room temperature and subsequently washed three times with PBS-T. Third, the prepared array is incubated for 90 minutes at room temperature with 50 ml of a 5 mg/l solution of the IgG form of GPC-8-10-57 in 1% BSA/PBS-T. Fourth, after binding, the membrane is washed three times with PBS-T and subsequently incubated for 1 hour at room temperature with a goat anti-human light chain antibody conjugated to horseradish peroxidase diluted 1/5000 in 1% BSA/PBS-T. Finally, the membrane is washed three times with PBS-T and any binding determined using chemiluminescence detection on X-ray film. As a control for unspecific binding of the goat anti-human light chain antibody, the peptide array is stripped by the following separate washings each at room temperature for 30 min: PBS-T (2 times), water, DMF, water, an aqueous solution containing 8M urea, 1% SDS, 0.5% DTT, a solution of 50% ethanol, 10% acetic acid in water (3 times each) and, finally, methanol (2 times). The membrane is again blocked, washed, incubated with goat anti-human I light chain antibody conjugated to horseradish peroxidase and developed as described above.

#### *7. Affinity of anti- HLA-DR antibody and antibody fragments*

In order to demonstrate the superior binding properties of anti-HLA antibody fragments of the invention, we measured their binding affinities to the human MHC class II DR protein (DRA\*0101/DRB1\*0401) using standard equipment employing plasmon resonance principles. Surprisingly, we achieved affinities in the sub-nanomolar range for IgG forms of certain anti-HLA-DR antibody fragments of the invention. For example, the affinity of the IgG forms of MS-GPC-8-27-41, MS-GPC-8-6-13 & MS-GPC-8-10-57 was measured as 0.3, 0.5 and 0.6 nM respectively (Table 3a). Also, we observed high affinities in the range of 2-8 nM for Fab fragments affinity matured at the CDR1 and CDR3 light chain regions (Table 3b). Fab fragments affinity matured at only the CDR3 light chain region showed affinities in the range of 40 to 100 nM (Table 3c), and even Fab fragments of non-optimised HuCAL antigen binding domains showed affinities in the sub  $\mu$ M range (Table 3d). Only a moderate increase in  $K_{on}$  (2-fold) was observed following CDR3 optimisation ( $K_{on}$  remained approximately constant throughout the antibody optimization process in the order of  $1 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ ), whilst a significant decrease in  $K_{off}$  was a surprising feature of the optimisation process – sub  $100 \text{ s}^{-1}$ , sub  $10 \text{ s}^{-1}$ , sub  $1 \text{ s}^{-1}$  and sub  $0.1 \text{ s}^{-1}$  for the

unoptimised Fabs, CDR3 optimised Fabs, CDR3/CDR1 optimised Fabs and IgG forms of anti-HLA-DR antibody fragments of the invention.

The affinities for anti-HLA antibody fragments of the invention were measured as follows. All measurements were conducted in HBS buffer (20mM HEPES, 150mM NaCl, pH7.4) at a flow rate of 20µl/min at 25°C on a BIAcore3000 instrument (Biacore AB, Sweden). MHC class II DR protein (prepared as example 1) was diluted in 100mM sodium acetate pH 4.5 to a concentration of 50 - 100 mg/ml, and coupled to a CM5 chip (Biacore AB) using standard EDC-NHS coupling chemistry with subsequent ethanolamine treatment as manufacturers directions. The coating density of MHCII was adjusted to between 500 and 4000 RU. Affinities were measured by injection of 5 different concentrations of the different antibodies and using the standard software of the Biacore instrument. Regeneration of the coupled surface was achieved using 10mM glycine pH2.3 and 7.5mM NaOH.

#### *8. Multivalent killing activity of anti HLA-DR antibodies and antibody fragments*

To demonstrate the effect of valency on cell killing, a cell killing assay was performed using monovalent, bivalent and multivalent compositions of anti-HLA-DR antibody fragments of the invention against GRANTA-519 cells. Anti-HLA-DR antibody fragments from the HuCAL library showed much higher cytotoxic activity when cross-linked to form a bivalent composition (60 – 90% killing at antibody fragment concentration of 200 nM) by co-incubation with anti-FLAG M2 mAb (Figure 3) compared to the monovalent form (5 – 30% killing at antibody fragment concentration of 200 nM). Incubation of cell lines alone or only in the presence of anti-FLAG M2 mAb without co-incubation of anti-HLA-DR antibody fragments did not lead to cytotoxicity as measured by cell viability. Treatment of cells as above but using 50 nM of the IgG4 forms (naturally bivalent) of the antibody fragments MS-GPC-8, MS-GPC-8-6-13, MS-GPC-8-10-57 and MS-GPC-8-27-41 without addition of anti-FLAG M2 mAb showed a killing efficiency after 4 hour incubation of 76%, 78%, 78% and 73% respectively.

Furthermore, we observed that higher order valences of the anti-HLA-DR antibody fragments further decrease cell viability significantly. On addition of Protein G to the incubation mix containing the IgG form of the anti-HLA-DR antibody fragments, the

multivalent complexes thus formed further decrease cell viability compared to the bivalent composition formed from incubation of the anti-HLA-DR antibody fragments with only the bivalent IgG form.

- 5 The killing efficiency of anti-HLA-DR antibody fragments selected from the HuCAL library was tested on the HLA-DR positive tumor cell line GRANTA-519 (DSMZ, Germany).  $2 \times 10^5$  cells were incubated for 4 h at 37°C under 6% CO<sub>2</sub> with 200 nM anti-HLA-DR antibody fragments in RPMI 1640 (PAA, Germany) supplemented with 2,5% heat inactivated FBS (Biowhittaker Europe, BE), 2mM L-glutamine, 1% non-  
10 essential amino acids, 1 mM sodium pyruvate and 0,1 mg/ml kanamycin. Each anti-HLA-DR antibody fragment was tested for its ability to kill activated tumor cells as a monovalent anti-HLA-DR antibody fragment or as a bivalent composition by the addition of 100 nM of a bivalent cross-linking anti-FLAG M2 mAb. After 4 h incubation at 37°C under 6% CO<sub>2</sub>, cell viability was determined by trypan blue staining and  
15 subsequent counting of remaining viable cells (Current Protocols in Immunology, 1997).

The above experiment was repeated using KARPAS-422 cells against a multivalent form of IgG forms of MS-GPC-8-10-57 and MS-GPC-8-27-41 prepared by a pre-  
20 incubation with a dilution series of the bacterial protein Protein G. Protein G has a high affinity and two binding sites for IgG antibodies, effectively cross-linking them to yield a total binding valency of 4. In a control using IgG alone without preincubation with Protein G, approximately 55% of cells were killed, while cell killing using IgG pre-  
incubated with Protein G gave a maximum of approximately 75% at a molar ratio of  
25 IgG antibody/Protein G of ~ 6 (based on a molecular weight of Protein G of 28.5 kD). Higher or lower molar ratios of IgG antibody/Protein G approached the cell killing efficiency of the pure IgG antibodies.

#### *9. Killing efficiency of anti-HLA-DR antibody fragments*

- 30 Experiments to determine the killing efficiency of the anti-HLA-DR cross-linked antibody fragments against other tumor cell lines that express HLA-DR molecules were conducted analogous to example 8. Tumor cell lines that show greater than 50% cell killing with the cross linked Fab form of MS-GPC-8 after 4 h incubation include MHH-CALL4, MN 60, BJAB, BONNA-12 which represent the diseases B cell acute

lymphoid leukemia, B cell acute lymphoid leukemia, Burkitt lymphoma and hairy cell leukemia respectively. Use of the cross-linked Fab form of the anti-HLA-DR antibody fragments MS-GPC-1, 6 and 10 also shows similar cytotoxic activity to the above tumor cell lines when formed as a bivalent agent using the cross-linking anti-FLAG  
5 M2 mAb.

The method described in example 8 was used to determine the maximum killing capacity for each of the cross-linked bivalent anti-HLA-DR antibody fragments against Priess cells. The maximum killing capacity observed for MS-GPC-1, MS-GPC-6, MS-  
10 GPC-8 & MS-GPC-10 was measured as 83%, 88%, 84% and 88% respectively. Antibody fragments generated according to example 4, when cross linked using anti-FLAG M2 mAb as above, also showed improved killing ability against GRANTA and Priess cells (Table 4).

15 *10. Killing efficiency of anti-HLA-DR IgG antibodies of human composition*

Compared to corresponding murine antibodies (Vidovic et al, 1995b; Nagy & Vidovic, 1996; Vidovic & Toral; 1998), we were surprised to observe significantly improved killing efficiency of IgG forms of certain anti-HLA-DR antibody fragments of the invention (Table 5). Following the method described in examples 8 and 9 but at 50  
20 nM, repeated measurements (3 to 5 replica experiments where cell number was counted in duplicate for each experiment) were made of the killing efficiency of the IgG forms of certain antibody fragments of the invention. When applied at a final concentration of only 50 nM, IgGs of the antibody fragments MS-GPC-8, MS-GPC-8-6-13, MS-GPC-8-10-57 & MS-GPC-8-27-41 killed more than 50% of cells from 16, 22,  
25 19 and 20 respectively of a panel of 24 human tumor cell lines that express HLA-DR antigen at a level greater than 10 fluorescent units as determined by example 11. Cells were treated with the two murine anti-HLA-DR antibodies L243 (Vidovic et al, 1995b) and 8D1 (Vidovic & Toral; 1998) at a significantly higher final concentration of mAb (200 nM), which reduced cell viability to a level below 50% viable cells in only 13  
30 and 12 of the 24 HLA-DR expressing cells lines, respectively. The cell line MHH-PREB-1 was singled out and not accounted as part of the panel of 24 cell lines despite its expression of HLA-DR antigen at a level greater than 10 fluorescent units due to the inability of any of the above antibodies to induce any significant reduction of cell viability. This is further explained in example 12.

Indeed, even at the significantly increased concentration, the two murine antibodies treated at 200 nM showed significantly less efficient killing compared to the IgG forms of anti-HLA DR antibody fragments of the invention. Not only do IgG forms of the human anti-HLA-DR antibody fragments of the invention show an overall increase in cell killing at lower concentrations compared to the murine antibodies, but they show less variance in killing efficiency across different cell lines. The coefficient of variance in killing for the human antibodies in this example is 32% (mean %killing = 68 +/- 22% (SD)), compared to over 62% (mean %killing = 49 +/- 31% (SD)) for the mouse antibodies. Statistically controlling for the effect on killing efficiency due to HLA expression by fitting logistic regression models to mean percentage killing against log(mean HLA DR expression) supports this observation (Figure 4). Not only is the fitted curve for the murine antibodies consistently lower than that for the human, but a larger variance in residuals from the murine antibody data (SD = 28%) is seen compared to the variance in residuals from the human antibody data (16%).

*11. Killing selectivity of antigen-binding domains against a human antigen for activated versus non-activated cells*

Human peripheral B cells were used to demonstrate that human anti-HLA-DR mAb-mediated cell killing is dependent on cell-activation. 50 ml of heparinised venous blood was taken from an HLA-DR typed healthy donor and fresh peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque Gradient Centrifugation (Histopaque-1077; Sigma) as described in Current Protocols in Immunology (John Wiley & Sons, Inc.; 1999). Purified B cells (~5% of peripheral blood leukocytes) were obtained from around  $5 \times 10^7$  PBMC using the B-cell isolation kit and MACS LS<sup>+</sup>/VS<sup>+</sup> columns (Miltenyi Biotec, Germany) according to manufacturers guidelines. Successful depletion of non-B cells was verified by FACS analysis of an aliquot of isolated B cells (HLA-DR positive and CD19 positive). Double staining and analysis is done with commercially available antibodies (BD Immunocytometry Systems, San Jose, CA, USA) using standard procedures as for example described in Current Protocols in Immunology (John Wiley & Sons, Inc.; 1999). An aliquot of the isolated B cells was tested for the ability of the cells to be activated by stimulation with Pokeweed mitogen (PWM) (Gibco BRL, Cat. No. 15360-019) diluted 1:25 in RPMI 1640 (PAA, Germany) supplemented with 10% FCS (Biowhittaker Europe, BE), 2mM



L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate and 0,1mg/ml kanamycin by incubation at 37°C under 6% CO<sub>2</sub> for three days. Successful activation was verified by FACS analysis of HLA-DR expression on the cell surface (Current Protocols in Immunology, John Wiley & Sons, Inc.; 1999).

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The selectivity for killing of activated cells versus non-activated cells was demonstrated by incubating 1x10<sup>6</sup>/ml B cells activated as above compared to non-activated cells, respectively with 50 nM of the IgG forms of MS-GPC-8-10-57, MS-GPC-8-27-41 or the murine IgG 10F12 (Vidovic et al., 1995b) in the medium described above but supplemented with 2,5% heat inactivated FCS instead of 10%, or with medium alone. After incubation at 37°C under 6% CO<sub>2</sub> for 1 or 4h, cell viability was determined by fluorescein diacetate staining (FDA) of viable and propidium iodide staining (PI) of dead cells and subsequent counting of the green (FDA) and red (PI) fluorescent cells using a fluorescence microscope (Leica, Germany) using standard procedures (Current Protocols in Immunology, 1997).

B cell activation was shown to be necessary for cell killing. In non-activated cells after 1 h of incubation with the anti-HLA-DR antibodies, the number of viable cells in the media corresponded to 81%, 117% 126% and 96% of the pre-incubation cell density for MS-GPC-8-10-57 (IgG), MS-GPC-8-27-41 (IgG), 10F12 and medium alone, respectively. In contrast, the number of viable activated B cells after 1 h incubation corresponded to 23%, 42% 83% and 66% of the pre-incubation cell density for MS-GPC-8-10-57 (IgG), MS-GPC-8-27-41 (IgG), 10F12 and medium alone, respectively. After 4 h of incubation, 78%, 83% 95% and 97% of the pre-incubation cell density for MS-GPC-8-10-57 (IgG), MS-GPC-8-27-41 (IgG), 10F12 and medium alone were found viable in non-activated cells, whereas the cell density had dropped to 23%, 24% 53% and 67% of the pre-incubation cell density for MS-GPC-8-10-57 (IgG), MS-GPC-8-27-41 (IgG), 10F12 and medium alone, respectively, in activated cells.

## 30 12. Killing activity of anti-HLA antibody fragments against the cell line MHH PreB 1

As evidenced in Table 5, we observed that our cross-linked anti-HLA-DR antibody fragments or IgGs did not readily kill a particular tumor cell line expressing HLA-DR at significant levels. We hypothesized that although established as a stable cell line, cells in this culture were not sufficiently activated. Therefore, we conducted an

experiment to stimulate activity of the MHH preB1 cell line, using increased cell-surface expression of HLA-DR molecule as a marker of activation as follows.

Non-adherently growing MHH preB1 cells were cultivated in RPMI medium containing the following additives (all from Gibco BRL and Bio Whittaker): 10% FCS, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 1x Kanamycin. Aliquots were activated to increase expression of HLA-DR molecule by incubation for one day with Lipopolysaccharide (LPS, 10 µg/ml), Interferon-gamma (IFN-γ, Roche, 40 ng/ml) and phyto-hemagglutinin (PHA, 5 µg/ml). The cell surface expression of HLA-DR molecules was monitored by flow cytometry with the FITC-conjugated mAb L243 (BD Immunocytometry Systems, San Jose, CA, USA). Incubation of MHH preB1 for one day in the presence of LPS, IFN- γ and PHA resulted in a 2-fold increase in HLA-DR surface density (mean fluorescence shift from 190 to 390). Cell killing was performed for 4 h in the above medium but containing a reduced FCS concentration (2.5%). A concentration series of the IgG forms of MS-GPC-8-27-41 & MS-GPC-8-10-57 was employed, consisting of final antibody concentrations of 3300, 550, 92, 15, 2.5, 0.42 and 0.07 nM, on each of an aliquot of non-activated and activated cells. Viable cells were identified microscopically by exclusion of Trypan blue. Whereas un-activated cell viability remains unaffected by the antibody up to the highest antibody concentration used, cell viability is dramatically reduced with increasing antibody concentration in activated MHH PreB1 cells (Figure 5).

### *13. Killing efficiency of anti-HLA-DR IgG antibodies of human composition against ex-vivo chronic lymphoid leukemia cells*

Using B cells isolated and purified from 10 patients suffering from chronic lymphoid leukemia (CLL), we demonstrated that IgG forms of anti-HLA-DR antibody fragments of the invention showed efficacy in killing of clinically relevant cells using an ex-vivo assay. B-cells were isolated and purified from 10 unrelated patients suffering from CLL (samples kindly provided by Prof Hallek, Ludwig Maximillan University, Munich) according to standard procedures (Buhmann et al., (1999)).  $2 \times 10^5$  cells were treated with 100 nM of IgG forms of the anti-HLA-DR antibody fragments MS-GPC-8, MS-GPC-8-10-57 or MS-GPC-8-27-41 and incubated for 4 or 24 hours analogous to examples 8 and 9. A replica set of cell cultures was established and activated by

incubation with HeLa-cells expressing CD40 ligand on their surface for three days before treatment with antibody (Buhmann et al., 1999). As controls, the murine IgG 10F12 (Vidovic et al., 1995b) or no antibody was used. Cell viability for each experiment was determined as described in example 12.

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Surprisingly, IgG forms of the anti-HLA-DR antibody fragments of the invention showed highly efficient and uniform killing - even across this diverse set of patient material. After only 4 hours of treatment, all three human IgGs gave a significant reduction in cell viability compared to the controls, and after 24 hours only 33% of  
10 cells remained viability (Figure 6). We found that on stimulating the ex-vivo cells further according to Buhmann et al (1999), the rate of killing was increased such that after only 4 hours culture with the human antibodies, only 24% of cells remained viable on average for all patient samples and antibody fragments of the invention.

15 *14. Determination of EC50 for anti-HLA-DR antibody fragments*

We demonstrated superior Effective Concentration at 50% effect (EC50) values in a cell-killing assay for certain forms of anti-HLA-DR antibody fragments selected from the HuCAL library compared to cytotoxic murine anti-HLA-DR antibodies (Table 6).

20 The EC50 for anti-HLA-DR antibody fragments selected from the HuCAL library were estimated using the HLA-DR positive cell line PRIESS or LG2 (ECACC, Salisbury UK).  $2 \times 10^5$  cells were incubated for 4 h at 37°C under 6% CO<sub>2</sub> in RPMI 1640 (PAA, Germany) supplemented with 2,5% heat inactivated FBS (Biowhittaker Europe, BE), 2mM L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate and  
25 0,1mg/ml kanamycin, together with dilution series of bivalent anti-HLA-DR antibody fragments. For the dilution series of Fab antibody fragments, an appropriate concentration of Fab fragment and anti-FLAG M2 antibody were premixed to generate bivalent compositions of the anti-HLA-DR antibody fragments. The concentrations stated refer to the concentration of bivalent composition such that the  
30 IgG and Fab EC50 values can be compared.

After 4 h incubation with bivalent antibody fragments at 37°C under 6% CO<sub>2</sub>, cell viability was determined by fluorescein diacetate staining and subsequent counting of remaining viable cells (Current Protocols in Immunology, 1997). Using standard

statistical software, non-linear logistic regression curves were fitted to replica data points and the EC50 estimated for each antibody fragment.

When cross-linked using the anti-FLAG M2 antibody, the Fab fragments MS-GPC-1,  
5 MS-GPC-8 & MS-GPC-10 selected from the HuCAL library (Example 4) showed an EC50 of less than 120 nM as expressed in terms of the concentration of the monovalent fragments, which corresponds to a 60 nM EC50 for the bivalent cross-linked (Fab)dimer-anti-Flag M2 conjugate. (Figure 7a). When cross-linked using the anti-FLAG M2 antibody, anti-HLA-DR antibody fragments optimised for affinity within  
10 the CDR3 region (Example 4) showed a further improved EC50 of less than 50 nM, or 25 nM in terms of the bivalent cross-linked fragment (Figure 7b), and those additionally optimised for affinity within the CDR1 region showed an EC50 of less than 30 nM (15 nM for bivalent fragment). In comparison, the EC50 of the cytotoxic murine anti-HLA-DR antibodies 8D1 (Vidovic & Toral; 1998) and L243 (Vidovic et al; 1995b)  
15 showed an EC50 of over 30 and 40 nM, respectively, within the same assay (Figure 7c).

Surprisingly, the IgG form of certain antibody fragments of the invention showed approximately 1.5 orders of magnitude improvement in EC50 compared to the murine  
20 antibodies (Figure 7d). For example, the IgG forms of MS-GPC-8-10-57 & MS-GPC-8-27-41 showed an EC50 of 1.2 and 1.2 nM respectively. Furthermore, despite being un-optimised for affinity, the IgG form of MS-GPC-8 showed an EC50 of less than 10 nM.

25 As has been shown in examples 11 and 12, the efficiency of killing of un-activated cells (normal peripheral B and MHH PreB cells respectively) is very low. After treatment with 50 nM of the IgG forms of MS-GPC-8-10-57 & MS-GPC-8-27-41, 78% and 83% of normal peripheral B cells, respectively, remain viable after 4 hours. Furthermore, at only 50nM concentration or either IgG, virtually 100% viability is seen  
30 for MHH PreB1 cells. Indeed, a decrease in the level of viability to below 50% cannot be achieved with these un-activated cells using reasonable concentration ranges (0.1 to 300 nM) of IgG or bivalent cross-linked Fab forms of the anti-HLA DR antibody fragments of the invention. Therefore, the EC50 for these un-activated cell types can be estimated to be at least 5 times higher than that shown for the non-optimised Fab

forms (EC<sub>50</sub> ~ 60 nM with respect to cross-linked bivalent fragment), and at least 10 times and 100 times higher than EC<sub>50</sub>s shown for the VHCDR3 optimised Fabs (~ 25 nM with respect to cross-linked bivalent fragment) and IgG forms of MS-GPC-8-10-57 (~1.2 nM) & MS-GPC-8-27-41 (~1.2 nM) respectively.

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### 15. Mechanism of cell-killing

The examples described above show that cell death occurs - needing only certain multivalent anti-HLA-DR antibody fragments to cause killing of activated cells. No further cytotoxic entities or immunological mechanisms were needed to cause cell death, therefore demonstrating that cell death is mediated through an innate pre-programmed mechanism of the activated cell. The mechanism of apoptosis is a widely understood process of pre-programmed cell death. We were surprised by certain characteristics of the cell killing we observed that suggested the mechanism of killing for activated cells when exposed to our human anti-HLA-DR antibody fragments was not what is commonly understood in the art as "apoptosis". For example, the observed rate of cell killing appeared to be significantly greater than the rate reported for apoptosis of immune cells (about 10 - 15 h; Truman et al., 1994). Two experiments were conducted to demonstrate that the mechanism of cell killing proceeded by a non-apoptotic mechanism.

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First, we used Annexin-V-FITC and propidium iodide (PI) staining techniques to distinguish between apoptotic and non-apoptotic cell death – cells undergoing apoptosis, "apoptotic cells", (Annexin-V positive/PI negative) can be distinguished from necrotic ("Dead") (Annexin-V positive/PI positive) and fully functional cells (Annexin-V negative/PI negative). Using the procedures recommended by the manufacturers of the AnnexinV and PI assays,  $1 \times 10^6$ /ml Priess cells were incubated at 37°C under 6% CO<sub>2</sub> with or without 200 nM anti-HLA-DR antibody fragment MS-GPC-8 together with 100 nM of the cross-linking anti-FLAG M2 mAb in RPMI 1640 (PAA, DE) supplemented with 2,5% heat inactivated FCS (Biowhittaker Europe, BE), 2mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 0,1 mg/ml kanamycin. To provide an apoptotic cell culture as control,  $1 \times 10^6$ /ml Priess cells were induced to enter apoptosis by incubation in the above medium at 37°C under 6% CO<sub>2</sub> with 50 µg/ml of the apoptosis-inducing anti-CD95 mAb DX2 (BD Pharmingen, Torrey Pine, CA, USA) cross-linked with 10 µg/ml Protein-G. At various

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incubation times (1, 15 and 60 min, 3 and 5 h) 200 µl samples were taken, washed twice and stained with Annexin-V-FITC (BD Pharmingen, Torrey Pine, CA, USA) and PI using Annexin-V binding buffer following the manufacturer's protocol. The amount of staining with Annexin-V-FITC and PI for each group of cells is analysed with a  
5 FACS Calibur (BD Immunocytometry Systems, San Jose, CA, USA).

Cell death induced through the cross-linked anti-HLA-DR antibody fragments shows a significantly different pattern of cell death than that of the anti-CD95 apoptosis inducing antibody or the cell culture incubated with anti-FLAG M2 mAb alone. The  
10 percentage of dead cells (as measured by Annexin-V positive/PI positive staining) for the anti-HLA-DR antibody fragment/anti-FLAG M2 mAb treated cells increases far more rapidly than that of the anti-CD95 or the control cells (Figure 8a). In contrast, the percentage of apoptotic cells (as measured by Annexin-V positive/PI negative staining) increases more rapidly for the anti-CD95 treated cells compared to the  
15 cross-linked anti-HLA-DR antibody fragments or the control cells (Figure 8b).

Second, we inhibited caspase activity using zDEVD-fmk, an irreversible Caspase-3 inhibitor, and zVAD-fmk, a broad spectrum Caspase inhibitor (both obtained from BioRad, Munich, DE). The mechanism of apoptosis is characterized by activity of  
20 caspases, and we hypothesized that if caspases were not necessary for anti HLA-DR mediated cell death, we would observe no change in the viability of cells undergoing cell death in the presence of these caspase inhibitors compared to those without.  $2 \times 10^5$  Priess cells were preincubated for 3 h at 37°C under 6% CO<sub>2</sub> with serial dilutions of the two caspase inhibitors ranging from 180 µM to 10 mM in RPMI 1640  
25 (PAA, DE) supplemented with 2,5% heat inactivated FCS (Biowhittaker Europe, BE), 2mM L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate and 0,1mg/ml kanamycin. HLA-DR mediated cell death was induced by adding 200 nM of the human anti-HLA-DR antibody fragment MS-GPC-8 and 100 nM of the cross-linking anti-M2 mAb. An anti-CD95 induced apoptotic cell culture served as a control  
30 for the activity of inhibitors (Drenou et al., 1999). After further incubation at 37°C and 6% CO<sub>2</sub>, cell viability after 4 and 24 h was determined by trypan blue staining and subsequent counting of non-stained cells. As we expected, cell viability of the anti-HLA-DR treated cell culture was not significantly modified by the presence of the Caspase inhibitors, while cell death induced through anti-CD95 treatment was

significantly decreased for the cell culture pre-incubated with the Caspase inhibitors. This observation supports our hypothesis that HLA-DR mediated cell death proceeds through a non-apoptotic mechanism that is independent of caspase proteases that can be inhibited by zDEVD-fm or zVAD-fmk.

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*16. In vivo therapy for cancer using an HLA-DR specific antibody*

We demonstrate that antigen-binding domains of human composition can successfully be used as a therapeutic for the treatment of cancer. Immunocompromised mice - such as scid, nude or Rag-1 knockout - are inoculated  
10 with a DR+ human lymphoma or leukemia cell line of interest. The tumor cell dose, usually  $1 \times 10^6$  to  $1 \times 10^7$ /mouse, is established for each tumor tested and administered subcutaneously (s.c.) or intravenously (i.v.). The mice are treated i.v. or s.c with the IgG form of the anti-HLA-DR antibody fragments MS-GPC-8, MS-GPC-8-10-57, MS-GPC-8-27-41 or others of the invention prepared as described above, using doses of  
15 1 to 25 mg/kg over 5 days. Survival of anti-HLA-DR treated and control untreated mice is monitored for up to 8 weeks after cessation of treatment. Tumor progression in the mice inoculated s.c. is additionally quantified by measuring tumor surface area. Significant prolongation of survival of up to 80% of anti-HLA-DR treated mice is observed during the experiment, and up to 50% mice survive at the end of the  
20 experiment. In s.c. inoculated and untreated mice, the tumor reaches a surface area of 2 - 3 cm<sup>2</sup>, while in anti-HLA-DR treated animals the tumor surface area is significantly less.

*17. Immunosuppression using anti-HLA-DR antibody fragments measured by  
25 reduction in IL-2 secretion*

We were surprised to observe that certain anti-HLA DR antibody fragments of the invention displayed substantial immunomodulatory properties within an assay measuring IL-2 secretion from immortalized T-cells. IgG forms of the antibody fragments MS-GPC-8-6-13, MS-GPC-8-10-57 & MS-GPC-8-27-41 showed very  
30 strong immunosuppressive properties in this assay with sub-nanomolar IC50 values and virtually 100% maximal inhibition (Figure 9a). Particularly surprising was our observation that certain monovalent compositions of the antibody fragments of the invention were able to strongly inhibit IL-2 secretion in the same assay. For example, Fab forms of the VHCDR3-selected and VLCDR3/VLCDR1 optimised antibody

fragments showed low single-digit nano-M IC<sub>50</sub>s and also almost 100% maximal inhibition (Figure 9b). Other monovalent anti-HLA DR antibody fragments of the invention showed significant immunosuppressive properties in the assay compared to control IgG and Fab fragments (Table 7).

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The immunomodulatory properties of anti-HLA DR antibody fragments was investigated by measuring IL-2 secretion from the hybridoma cell line T-Hyb 1 stimulated using DR-transgenic antigen presenting cells (APC) under conditions of half-maximal antigen stimulation. IL-2 secretion was detected and measured using a standard ELISA method provided by the OptiEIA mouse IL-2 kit of Pharmingen (Torrey Pine, CA, USA). APCs were isolated from the spleen of unimmunized chimeric 0401-IE transgenic mice (Ito et al. 1996) according to standard procedures. 1.5x10<sup>5</sup> APCs were added to 0.2 ml wells of 96-well in RPMI medium containing the following additives (all from Gibco BRL and PAA): 10 % FCS, 2mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 0.1 g/l kanamycin. Hen egg ovalbumin was added to a final concentration of 200 µg/ml in a final volume of 100 µl of the above medium, the cells incubated with this antigen for 30 min at 37°C under 6% CO<sub>2</sub>. Anti-HLA DR antibody fragments were added to each well at various concentrations (typically in a range from 0.1 to 200 nM), the plate incubated for 1 h at 37°C/6% CO<sub>2</sub> and 2x10<sup>5</sup> T-Hyb 1 cells added to give a final volume of 200 µl in the above medium. After incubation for 24 h, 100 µl of supernatant was transferred to an ELISA plate (Nunc-Immuno Plate MaxiSorp surface, Nunc, Roskilde, DK) previously coated with IL-2 Capture Antibody (BD Pharmingen, Torrey Pine, CA, USA), the amount of IL-2 was quantified according to the manufacturer's directions using the OptiEIA Mouse IL-2 kit and the plate read using a Victor V reader (Wallac, Finland). Secreted IL-2 in pg/ml was calibrated using the IL-2 standards provided in the kit.

The T-cell hybridoma line T-Hyb1 was established by fusion of a T-cell receptor negative variant of the thymoma line BW 5147 (ATCC) and lymph node cells from chimeric 0401-IE transgenic mice previously immunized with hen egg ovalbumin (Ito et al. 1996). The clone T-Hyb1 was selected for the assay since it responded to antigen specific stimulation with high IL-2 secretion.



*18. Immunosuppression using an HLA-DR specific antibody measured by T cell proliferation*

Immunomodulatory properties of the anti-HLA DR antibody fragments were also seen within an assay that measures T cell proliferation. The IC<sub>50</sub> value for inhibition of T cell proliferation of the IgG form of MS-GPC-8-10-57 and MS-GPC-8-27-41 were 11 and 20 nM respectively (Figure 10). The anti-HLA DR antibody fragments were tested as follows to inhibit the proliferative T cell response of antigen-primed lymph node cells from mice carrying a chimeric mouse-human class II transgene with an RA-associated peptide binding site, and lack murine class II molecules (Muller et al., 1990; Woods et al., 1994; Current Protocols in Immunology, Vol. 2, 7.21; Ito et al., 1996). Here, the immunization takes place *in vivo*, but the inhibition and readout are *ex vivo*. Transgenic mice expressing MHC class II molecules with binding sites of the RA associated molecule, DRB\*0401 were commercially obtained. These mice lack murine MHC class II, and thus, all Th responses are channelled through a single human RA-associated MHC class II molecule (Ito et al. 1996). These transgenic mice represent a model for testing human class II antagonists.

The inhibitory effect of the anti-HLA-DR antibody fragments and their IgG forms were tested on T-cell proliferation measured using chimeric T-cells and antigen presenting cells isolated from the lymph nodes of chimeric 0401-IE transgenic mice (Taconic, USA) previously immunized with hen egg ovalbumin (Ito et al. 1996) according to standard procedures.  $1.5 \times 10^5$  cells are incubated in 0.2 ml wells of 96-well tissue culture plates in the presence of ovalbumin (30 µg per well - half-maximal stimulatory concentration) and a dilution series of the anti-HLA DR antibody fragment or IgG form under test (0.1 nM - 200 nM) in serum free HL-1 medium containing 2 mM L-glutamine and 0.1 g/l Kanamycin for three days. Antigen specific proliferation is measured by <sup>3</sup>H-methyl-thymidin(1 µCi/well) incorporation during the last 16h of culture (Falcioni et al., 1999). Cells are harvested, and <sup>3</sup>H incorporation measured using a scintillation counter (TopCount, Wallac Finland). Inhibition of T-cell proliferation on treatment with the anti-HLA DR antibody fragment and its IgG form may be observed by comparison to control wells containing antigen.

19. *Selection of useful polypeptide for the treatment of cancers*

In order to select the most appropriate protein/peptide to enter further experiments and to assess its suitability for use in a therapeutic composition for the treatment of cancers, additional data are collected. Such data for each IgG form of the anti-HLA antigen antibody fragments can include the binding affinity, *in vitro* killing efficiency as measured by EC50 and cytotoxicity across a panel of tumor cell lines, the maximal percentage cell killing as estimated *in vitro*, and tumor reduction data and mouse survival data from *in vivo* animal models.

- 10 The IgG form of the anti-HLA antigen antibody fragments that shows the highest affinity, the lowest EC50 for killing, the highest maximal percentage cell killing and broadest across various tumor cell lines, the best tumor reduction data and/or the best mouse-survival data may be chosen to enter further experiments. Such experiments may include, for example, therapeutic profiling and toxicology in animals and phase I clinical trials in humans.

20. *Selection of useful polypeptide for the treatment of diseases of the immune system*

In order to select the most appropriate protein/peptide to enter further experiments and to assess its suitability for use in a therapeutic composition for the treatment of diseases of the immune system, additional data are collected. Such data for each monovalent antibody fragment or IgG form of the anti-HLA antigen antibody fragments can include the affinity, reactivity, specificity, IC50-values, for inhibition of IL-2 secretion and of T-cell proliferation, or *in vitro* killing efficiency as measured by EC50 and the maximal percentage cell killing as estimated *in vitro*, and DR-transgenic models of transplant rejection and graft vs. host disease.

- 30 The antibody fragment or IgG form of the anti-HLA antigen antibody fragments that shows the lowest EC50, highest affinity, highest killing, best specificity and/or greatest inhibition of T-cell proliferation or IL-2 secretion, and high efficacy in inhibiting transplant rejection and/or graft vs. host disease in appropriate models, might be chosen to enter further experiments. Such experiments may include, for example, therapeutic profiling and toxicology in animals and phase I clinical trials in humans.

**Table 1:**  
VH and VL families, VL CDR1 and VH/VL CDR 3 sequences of HLA-DR-specific polypeptides

Clone	VH	CDR3 Length	VH-CDR3-Seq.	VL	VL-CDR1-Seq.	CDR3 Length	VL-CDR3-Seq.	Families
MS-GPC-1	H2	10	QYGHRRGGFDH	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDFNES	H2 $\lambda$ 1
MS-GPC-6	H3	9	GYGRYSPDL	K3	RASQSVSSSYLA	8	QQYSNLPF	H3 K 3
MS-GPC-8	H2	10	SPRYRGAFDY	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDMPQA	H2 $\lambda$ 1
MS-GPC-10	H2	10	QLHYRGGFDL	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDLTMG	H2 $\lambda$ 1
MS-GPC-8-1	H2	10	SPRYRGAFDY	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDFSHY	H2 $\lambda$ 1
MS-GPC-8-6	H2	10	SPRYRGAFDY	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDYDHY	H2 $\lambda$ 1
MS-GPC-8-9	H2	10	SPRYRGAFDY	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDIQLH	H2 $\lambda$ 1
MS-GPC-8-10	H2	10	SPRYRGAFDY	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDLIRH	H2 $\lambda$ 1
MS-GPC-8-17	H2	10	SPRYRGAFDY	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDFSIV	H2 $\lambda$ 1
MS-GPC-8-18	H2	10	SPRYRGAFDY	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDFSIV	H2 $\lambda$ 1
MS-GPC-8-27	H2	10	SPRYRGAFDY	$\lambda$ 1	SGSSSNIGSNYVS	8	QSYDMNVH	H2 $\lambda$ 1

[illegible]

Table 2:

Steps in Antibody optimisation	Fab	$k_{on} [s^{-1}M^{-1}] \times 10^5$ +/- SD	$k_{off} [s^{-1}] \times 10^{-3}$ +/- SD	$K_D [nM]$ +/- SD	L-CDR3	L-CDR1
Parental Fab	MS-GPC-8	$0.99 \pm 0.40$	$29.0 \pm 8.40$	$346.1 \pm 140.5^a$	QSYDMPQA	SGSSSNIGSNYVS
L-CDR3-optim.	-8-1	1.93	20.9	$108^e$		
L-CDR3-optim.	-8-6	$0.96 \pm 0.14$	$5.48 \pm 0.73$	$58.6 \pm 11.7^b$		
L-CDR3-optim.	-8-9	1.85	16.6	$90.1^e$		
L-CDR3-optim.	-8-10	nd	$7.0^e$	nd		
L-CDR3-optim.	-8-17	1.0	5.48	$54.7^e$		
L-CDR3-optim.	-8-18	1.06	8.3	$78.3^e$		
L-CDR3-optim.	-8-27	nd	$6.6^e$	nd		
L-CDR3-optim.	-8-6	$0.96 \pm 0.14$	$5.48 \pm 0.73$	$58.6 \pm 11.7^b$	QSYDYDHY	SGSSSNIGSNYVS
L-CDR3+1-opt.	-8-6-2	$1.23 \pm 0.11$	$0.94 \pm 0.07$	$7.61 \pm 0.25^c$	QSYDYDHY	SGSESNIGSNYVH
L-CDR3+1-opt.	-8-6-19	$1.10 \pm 0.08$	$0.96 \pm 0.15$	$8.74 \pm 1.33^c$	QSYDYDHY	SGSESNIGSNYVA
L-CDR3+1-opt.	-8-6-27	$1.80 \pm 0.24$	$1.10 \pm 0.15$	$6.30 \pm 0.63^d$	QSYDYDHY	SGSDSNIGSNYVT
L-CDR3+1-opt.	-8-6-45	$1.20 \pm 0.07$	$1.03 \pm 0.04$	$8.63 \pm 0.61^c$	QSYDYDHY	SGSEPNIGSNYVF
L-CDR3+1-opt.	-8-6-13	$1.90 \pm 0.26$	$0.55 \pm 0.05$	$2.96 \pm 0.46^c$	QSYDYDHY	SGSESNIGSNYVT
L-CDR3+1-opt.	-8-6-47	$1.97 \pm 0.29$	$0.62 \pm 0.04$	$3.18 \pm 0.33^c$	QSYDYDHY	SGSESNIGSNYVS
L-CDR3+1-opt.	-8-10-57	$1.65 \pm 0.21$	$0.44 \pm 0.06$	$2.67 \pm 0.25^c$	QSYDLIRH	SGSESNIGSNYVQ
L-CDR3+1-opt.	-8-27-7	$1.74 \pm 0.21$	$0.57 \pm 0.07$	$3.30 \pm 0.34^d$	QSYDMNVH	SGSESNIGSNYVG
L-CDR3+1-opt.	-8-27-10	$1.76 \pm 0.21$	$0.53 \pm 0.05$	$3.01 \pm 0.21^c$	QSYDMNVH	SGSESNIGSNYVN

L-CDR3+1-opt.	-8-27-41	1.67 ± 0.16	0.49 ± 0.03	2.93 ± 0.27 <sup>d)</sup>	QSYDMNVH	SGSESIGNNYVQ
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- a) Affinity data of MS-GPC-8 are based on 8 different Fab-preparations which were measured on 4 different chips (2 x 500, 1000, 4000RU)
- b) For MS-GPC-8-6 mean and standard deviation of 3 different preparations on 3 different chips (500, 4000, 3000RU) is shown.
- c) 3000RU MHCII were immobilized on a CM5-chip. For each measurement 7 different concentrations from 1µM to 16nM were injected on the surface. Dissociation time: 150sec, regeneration was reached by 6µl 10mM Glycine pH2.3 followed by 8µl 7.5mM NaOH. For MS-GPC-8-6-19 mean and standard deviation of 4 different preparations are shown whereas for all other binders mean and standard deviation of 3 different preparations are shown.
- d) One protein preparation is measured on 3 different chips (3000, 2800 and 6500RU).
- e) Affinity determination of matured MHCII binder on a 4000RU density chips; single measurement.
- Molecular weights were determined after size exclusion chromatography and found 100% monomeric with the right molecular weight between 45 and 48 kDa.

**Table 3a**

Affinities of selected IgG4 monoclonal antibodies constructed from F<sub>ab</sub>'s. Errors represent standard deviations

Binder (IgG <sub>4</sub> )	k <sub>on</sub> [M <sup>-1</sup> s <sup>-1</sup> ] x10 <sup>5</sup>	k <sub>off</sub> [s <sup>-1</sup> ] x10 <sup>-5</sup>	K <sub>D</sub> [nM]
MS-GPC-8-27-41	1.1 ± 0.2	3,1 ± 0.4	0,31 ± 0.06
MS-GPC-8-6-13	0,7 ± 0.1	3 ± 1	0,5 ± 0.2
MS-GPC-8-10-57	0,7 ± 0.2	4 ± 1	0,6 ± 0.2

**Table 3b**

Affinities of binders obtained out of affinity maturation of CDR1 light chain optimisation following CDR3 heavy chain optimisation. Errors represent standard deviations

Binder (F <sub>ab</sub> )	k <sub>on</sub> [M <sup>-1</sup> s <sup>-1</sup> ] x10 <sup>5</sup>	k <sub>off</sub> [s <sup>-1</sup> ] x10 <sup>-3</sup>	K <sub>D</sub> [nM]
MS-GPC-8-6-2	1.2 ± 0.1	0.94 ± 0.07	7.6 ± 0.3
MS-GPC-8-6-19	1.1 ± 0.1	1.0 ± 0.2	9 ± 1
MS-GPC-8-6-27	1.8 ± 0.2	1.1 ± 0.2	6.3 ± 0.6
MS-GPC-8-6-45	1.20 ± 0.07	1.03 ± 0.04	8.6 ± 0.6
MS-GPC-8-6-13	1.9 ± 0.3	0.55 ± 0.05	3.0 ± 0.5
MS-GPC-8-6-47	2.0 ± 0.3	0.62 ± 0.04	3.2 ± 0.3
MS-GPC-8-10-57	1.7 ± 0.2	0.44 ± 0.06	2.7 ± 0.3
MS-GPC-8-27-7	1.7 ± 0.2	0.57 ± 0.07	3.3 ± 0.3
MS-GPC-8-27-10	1.8 ± 0.2	0.53 ± 0.05	3.0 ± 0.2
MS-GPC-8-27-41	1.7 ± 0.2	0.49 ± 0.03	2.9 ± 0.3

**Table 3c**

Binders obtained out of affinity maturation of GPC8 by CDR3 light chain optimisation

Binder ( $F_{ab}$ )	$k_{on} [M^{-1}s^{-1}] \times 10^5$	$k_{off} [s^{-1}] \times 10^{-3}$	$K_D [nM]$
MS-GPC 8-18	1.06	8.3	78.3
MS-GPC 8-9	1.85	16.6	90.1
MS-GPC 8-1	1.93	20.9	108
MS-GPC 8-17	1.0	5.48	54.7
MS-GPC-8-6 <sup>a)</sup>	1.2 +/- 0.1	5.5 +/- 0.7	8 +/- 12

Chip density 4000RU MHCII

a) For MS-GPC-8-6 mean and standard deviation of 3 different preparations on 3 different chips (500, 4000, 3000RU) is shown.

**Table 3d**

Binders obtained out of HuCAL in scFv form and their converted Fabs

Binder	scF <sub>v</sub>			F <sub>ab</sub>		
	$k_{on} [M^{-1}s^{-1}] \times 10^5$	$k_{off} [s^{-1}] \times 10^{-3}$	$K_D [nM]$	$k_{on} [M^{-1}s^{-1}] \times 10^5$	$k_{off} [s^{-1}] \times 10^{-3}$	$K_D [nM]$
MS-GPC 1	0.413	61	1500	0.639	53	820
MS-GPC 6	0.435	200	4600	0.135	114	8470 (1 curve)
MS-GPC 8	0.114	76	560	0.99 +/- 0.40	29.0 +/- 8.4	346 <sup>a)</sup> +/- 141
MS-GPC 10	0.187	180	9625	0.22	63	2860

Chip density 500RU MHCII

a) Affinity data of MS-GPC-8 are based on 8 different Fab-preparations which were measured on 4 different chips (2 x 500, 1000, 4000RU) and are shown with standard deviation.



## Table 4

Killing efficiency after 4 hour incubation of cells with cross-linked anti-HLA-DR antibody fragments, and maximum killing after 24 hour incubation

Cross-linked Fab fragment	Killing efficiency against GRANTA	Maximum killing against Priess
MS-GPC-1	+	+
MS-GPC-6	+	+
MS-GPC-8	+	+
MS-GPC-10	+	+
MS-GPC-8-6	++	++
MS-GPC-8-17	++	++
MS-GPC-8-6-13	+++	+++
MS-GPC-8-10-57	+++	+++
MS-GPC-8-27-41	+++	+++

**Table 5**

Killing efficiency of anti-HLA-DR IgG antibodies of human composition compared to murine anti-HLA-DR antibodies against a panel of lymphoid tumor cell lines.

Cell line			HLA-DR expression mean-FL	% Killing by mAb					
Name	DR type	Type		murine mAbs		human mAbs			
				L243	8D1	MS-GPC-8	8-27-41	8-10-57	8-6-13
LG-2	1,1	B-lymphoblastoid	458	79	85	86	87	88	82
Priess	4,4	B-lymphoblastoid	621	87	83	85	88	93	74
ARH-77	12	B-lymphoblastoid	301	88	73	84	85	88	87
GRANTA-519	2,11	B cell non-Hodgkin	1465	83	56	76	78	78	73
KARPAS-422	2,4	B cell non-Hodgkin	186	25	32	51	66	68	71
KARPAS-299	1,2	T cell non-Hodgkin	919	78	25	81	82	79	76
DOHH-2	1,2	B cell lymphoma	444	29	23	58	59	60	53
SR-786	1,2	T cell lymphoma	142	3	8	1	53	44	26
MHH-CALL-4	1,2	B-ALL	348	35	41	43	63	46	43
MN-60	10,13	B-ALL	1120	46	22	71	69	66	67
BJAB	12,13	Burkitt lymph.	338	53	59	49	71	67	64
RAJI	10, 17	Burkitt lymph.	617	69	64	81	84	86	83
L-428	12	Hodgkin's lymph.	244	82	81	82	91	91	92

HDLM-2	Hodgkin's lymph.	326	77	73	89	88	84	90
HD-MY-Z	Hodgkin's lymph.	79	35	39	49	69	57	72
KM-H2	Hodgkin's lymph.	619	81	56	75	86	88	87
L1236	Hodgkin's lymph.	41	52	62	44	63	66	66
BONNA-12	hairy cell leuk.	2431	92	91	91	92	91	86
HC-1	hairy cell leuk.	372	88	89	89	93	86	93
NALM-1	CML	1078	44	4	83	82	78	65
L-363	plasma cell leu.	49	6	5	26	26	24	19
EOL-1	AML (eosinophil)	536	22	13	36	69	49	53
LP-1	multiple myeloma	315	12	0	61	73	70	73
RPMI-8226	multiple myeloma	19	6	0	14	29	26	19
MHH-PREB-1	B cell non-Hodgkin	175	3	3	2	4	8	11
MHH-CALL-2	B cell precursor leu.	+	5	5				
OPM-2	multiple myeloma	3	13	0	8	1	4	5
KASUMI-1	AML	5	0	0	8	10	10	6
HL-60	AML	3	18	0	3	15	9	22
LAMA-84	CML	7	7	9	5	11	5	7

% Killing: 100 - % viable cells after a 4h treatment with 200 nM murine or 50 nM human mAb at 37°C.

**Table 6**

EC50 values for certain anti-HLA-DR antibody fragments of the invention in a cell-killing assay against lymphoid tumor cells. All EC50 refer to nanomolar concentrations of the bivalent agent (IgG or cross-linked Fab) such that values for cross-linked Fab and IgG forms can be compared.

Antibody fragment	Form	Cell line tested	EC50 of cell killing (nM) +/- SE for bivalent agent
MS-GPC-1	Fab	PRIESS	54 ± 14
MS-GPC-8	Fab	PRIESS	31 ± 9
MS-GPC-10	Fab	PRIESS	33 ± 5
MS-GPC-8-17	Fab	PRIESS	16 ± 4
MS-GPC-8-6-2	Fab	PRIESS	8 ± 2
MS-GPC-8-10-57	Fab	LG2	7.2
MS-GPC-8-27-41	Fab	LG2	7.2
MS-GPC-8-27-41	Fab	PRIESS	7.7
MS-GPC-8	IgG4	PRIESS	8.3
MS-GPC-8-27-41	IgG4	PRIESS	1.1 ± 0.1
MS-GPC-8-10-57	IgG4	PRIESS	1.1 ± 0.2
MS-GPC-8-27-41	IgG4	LG2	1.23 ± 0.2
MS-GPC-8-10-57	IgG4	LG2	1.0 ± 0.1
8D1	mIgG	PRIESS	33
L243	mIgG	PRIESS	47

**Table 7**

IC50 values for certain anti-HLA-DR antibody fragments of the invention in an assay to determine IL-2 secretion after antigen-specific stimulation of T-Hyb 1 cells. IC50 for the IgG forms (bivalent) are represented as molar concentrations, while in order to provide easy comparison, IC50s for the Fab forms (monovalent) are expressed in terms of half the concentration of the Fab to enable direct comparison to IgG forms.

Anti-HLA-DR antibody fragment	Form	IC50 (IgG/nM) ((Fab)/2/nM)		Maximum inhibition(%)
		Mean	SE	
MS-GPC-8-10-57	IgG	0.31	0.01	100
MS-GPC-8-27-41	IgG	0.28	0.07	100
MS-GPC-8-6-13	IgG	0.42	0.06	100
MS-GPC-8-6-2	IgG	3.6	1.1	100
MS-GPC-8-6	IgG	6.7	2.0	100
MS-GPC-8	IgG	11.0	0.8	100
MS-GPC-8-6-2	Fab	4.7	1.9	100
MS-GPC-8-6-13	Fab	2.1	0.8	100
MS-GPC-8-6-19	Fab	5.3	0.2	100
MS-GPC-8-10-57	Fab	2.9	1.0	100
MS-GPC-8-6-27	Fab	3.0	1.2	100
MS-GPC-8-6-47	Fab	2.6	0.6	100
MS-GPC-8-27-7	Fab	5.9	2.2	100
MS-GPC-8-27-10	Fab	7.3	1.9	100
MS-GPC-8-27-41	Fab	3.6	0.7	100
MS-GPC-8-6	Fab	20		100
MS-GPC-8	Fab	110		100

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## Claims

1. A composition including a polypeptide comprising an antibody-based antigen-binding domain of human composition with binding specificity for an antigen expressed on the surface of a human cell, wherein treating cells expressing said antigen with a multivalent polypeptide having two or more of said antigen-binding domains causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing.
2. A composition including a polypeptide comprising an antibody-based antigen-binding domain which binds to human HLA DR with a  $K_d$  of  $1\mu\text{M}$  or less, wherein treating cells expressing HLA DR with a multivalent polypeptide having two or more of said antigen-binding domains causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing.
3. A composition including a multivalent polypeptide comprising a plurality of antibody-based antigen-binding domains of human composition which specifically bind to human HLA DR, wherein treating cells expressing HLA DR with said multivalent polypeptide causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing, wherein said antigen-binding domains individually bind to human HLA DR with a  $K_d$  of  $1\mu\text{M}$  or less.
4. A composition including a multivalent polypeptide comprising a plurality of antibody-based antigen-binding domains of human composition which specifically bind to human HLA DR, wherein treating cells expressing HLA DR with said multivalent polypeptide causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said cell killing, wherein said multivalent polypeptide has an  $\text{EC}_{50}$  of 100 nM or less for killing activated lymphoid cells.
5. A composition including a polypeptide comprising at least one antibody-based antigen-binding domain that binds to human HLA DR with a  $K_d$  of  $1\mu\text{M}$  or less, said antigen-binding domain being isolated by a method which includes isolation of VL and VH domains of human composition from a recombinant antibody library by ability to bind to at least one epitope of human HLA DR, wherein treating cells expressing HLA DR with a multivalent polypeptide having two or more of said antigen binding domains causes or leads to killing of said cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing.

6. The composition of claim 5, wherein the method for isolating the antigen-binding domain includes the further steps of:
  - a. generating a library of variants of at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
  - b. isolation of VL and VH domains from the library of variants by ability to bind to human HLA DR with a  $K_d$  of 1  $\mu$ M or less.
7. The composition of any of claims 1-6, wherein the multivalent polypeptide has an  $EC_{50}$  for killing transformed cells at least 5-fold lower than the  $EC_{50}$  for killing normal cells.
8. The composition of any of claims 1-6, wherein the multivalent polypeptide has an  $EC_{50}$  for killing activated cells at least 5-fold lower than the  $EC_{50}$  for killing unactivated cells.
9. The composition of any of claims 1-6, wherein the multivalent polypeptide has an  $EC_{50}$  of 50nM or less for killing transformed cells.
10. The composition of any of claims 1-6, wherein the multivalent polypeptide has an  $EC_{50}$  for killing lymphoid tumor cells of 10nM or less.
11. The composition of any of claim 1-6 or 8, wherein the multivalent polypeptide kills activated lymphoid cells.
12. The composition of claim 11, wherein said activated lymphoid cells are lymphoid tumor cells representing a disease selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma, chronic myeloid leukemia, chronic lymphoid leukemia, and multiple myeloid leukemia.
13. The composition of claim 11, wherein said activated lymphoid cells are from a cell line taken from the list of Priess, GRANTA-519, KARPAS-422, KARPAS-299, DOHH-2, SR-786, MHH-CALL-4, MN-60, BJAB, RAJI, L-428, HDLM-2, HD-MY-Z, KM-H2, L1236, BONNA-12, HC-1, NALM-1, L-363, EOL-1, LP-1, RPMI-8226, and MHH-PREB-1 cell lines.
14. The composition of any of claims 1-6, wherein the multivalent polypeptide has an  $EC_{50}$  of 100nM or less for killing cells of at least one of lymphoid tumor cell lines selected from the list of KARPAS-422, DOHH-2, SR-7, MHH-CALL-4, MN-60, HD-MY-Z, NALM-1 and LP-1.

15. The composition of any of claims 1-6, wherein the multivalent polypeptide has an  $EC_{50}$  of 50nM or less for killing cells from at least one lymphoid tumor cell line selected from the list of KARPAS-422, DOHH-2, MN-60, NALM-1 and LP-1.
16. The composition of any of claims 1-6, wherein the multivalent polypeptide has an  $EC_{50}$  of 10nM or less for killing cells from at least one B cell lymphoblastoid cell line selected from the list LG2 and Priess.
17. The composition of any of claims 1-6, wherein said cells are non-lymphoid cells that express MHC class II molecules
18. The composition of any of claims 1-6, wherein said antigen-binding domain binds to the  $\beta$ -chain of HLA-DR.
19. The composition of claim 18, wherein said antigen-binding domain binds to the first domain of the  $\beta$ -chain of HLA-DR.
20. The composition of any of claims 1-6, wherein said antigen-binding domain binds to one or more HLA-DR types selected from the group consisting of DR1-0101, DR2-15021, DR3-0301, DR4Dw4-0401, DR4Dw10-0402, DR4Dw14-0404, DR6-1302, DR6-1401, DR8-8031, DR9-9012, DRw53-B4\*0101 and DRw52-B3\*0101.
21. The composition of claim 20, wherein said antigen-binding domain binds to at least 5 different of said HLA-DR types.
22. The composition of any one of claims 1-6, wherein said antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.
23. The composition of any one of claims 1-6, wherein said antigen-binding domain includes of a combination of HuCAL VH2 and HuCAL V $\lambda$ 1, wherein the VH CDR3, VL CDR1 And VL CDR3 is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

24. The composition of any one of claims 1-23, wherein said antigen-binding domain includes a combination of HuCAL VH2 and HuCAL Vλ1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and/or

wherein the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

wherein each n independently represents any amino acid residue.

25. The composition of claim 24, wherein the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.
26. The composition of any one of claims 1-23, wherein said antigen-binding domain competes for antigen binding with an antibody including a combination of HuCAL VH2 and HuCAL Vλ1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

each n independently represents any amino acid residue; and/or

the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

each n independently represents any amino acid residue.

27. The composition of claim 26, wherein the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.

28. The composition of any one of claims 1-27, wherein said antigen-binding domain includes a VL CDR1 sequence represented in the general formula

SGSnnNIGnNYVn

wherein each n independently represents any amino acid residue.

29. The composition of claim 28, wherein the CDR1 sequence is SGSESNIGNNYVQ.

30. The composition of any of claims 1-29, wherein the mechanism of said killing involves an innate pre-programmed process of said cell.
31. The composition of claim 30, wherein said killing is non-apoptotic.
32. The composition of claim 30, wherein said killing is dependent on the action of non-caspase proteases, and/or wherein said killing cannot be inhibited by zVAD-fmk or zDEVD-fmk.
33. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide including at least a F(ab')<sub>2</sub> antibody fragment or a mini-antibody fragment.
34. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide comprising at least two monovalent antibody fragments selected from Fv, scFv, dsFv and Fab fragments, and further comprises a cross-linking moiety or moieties.
35. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide comprising at least one full antibody selected from the antibodies of classes IgG1, 2a, 2b, 3, 4, IgA, and IgM.
36. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide that is formed prior to binding to a cell.
37. The composition of any one of claims 1-32, wherein said antibody-based antigen-binding domain is part of a multivalent polypeptide that is formed after binding to a cell.
38. The composition of claim 3 or 4, wherein the antigen binding sites are cross-linked to a polymer.
39. A nucleic acid comprising a protein coding sequence for an antigen-binding domain comprised in any of claims 1-32, or a multivalent polypeptide thereof.
40. A vector comprising the nucleic acid of claim 39, and a transcriptional regulatory sequence operably linked thereto.
41. A host cell harboring at least one nucleic acid of claim 39 or the vector of claim 40.
42. A method for the production of composition comprising a multivalent polypeptide that causes or leads to killing of cells in a manner where neither cytotoxic entities nor immunological mechanisms are needed for said killing, comprising culturing the cells

- of claim 41 under conditions wherein the nucleic acid is expressed either as a multivalent polypeptide or as a polypeptide comprising at least one antigen binding domains which is subsequently treated to form a multivalent polypeptide composition.
43. The composition of any of claims 1-38, formulated in a pharmaceutically acceptable carrier and/or diluent.
  44. The use of a composition of any of claims 1-38, for preparing a pharmaceutical preparation for the treatment of animals.
  45. The use of a nucleic acid of claim 39 for preparing a pharmaceutical preparation for the treatment of animals
  46. The use of a host cell of claim 41 for preparing a pharmaceutical preparation for the treatment of animals
  47. The use of the method of claim 42 for preparing a pharmaceutical preparation for the treatment of animals
  48. The use according to claim 44-47, wherein said animal is a human.
  49. The use according to claim 44-48, for the treatment of cell proliferative disorders, wherein said antibody-based antigen binding domain is part of a multivalent polypeptide.
  50. The use according to claim 49, wherein said treatment is the treatment of disorders involving transformed cells expressing MHC class II antigens.
  51. The use according claim 49 or 50, wherein said treatment is the treatment of a disorder selected from B cell non-Hodgkin lymphoma, B cell lymphoma, B cell acute lymphoid leukemia, Burkitt lymphoma, Hodgkin lymphoma, hairy cell leukemia, acute myeloid leukemia, T cell lymphoma, T cell non-Hodgkin lymphoma, chronic myeloid leukemia, chronic lymphoid leukemia, and multiple myeloid leukemia.
  52. The use according to any of claims 44-48, wherein said treatment is the treatment of disorders involving unwanted activation of cells of the immune system, such as lymphoid cells expressing MHC class II.
  53. The use according to any of claims 44-48, wherein said treatment is the treatment of a disorder selected from rheumatoid arthritis, juvenile arthritis, multiple sclerosis, Grave's disease, insulin-dependent diabetes, narcolepsy, psoriasis, systemic lupus erythematosus, ankylosing spondylitis, transplant rejection, graft vs. host disease, Hashimoto's disease, myasthenia gravis, pemphigus vulgaris, glomerulonephritis, thyroiditis, pancreatitis, insulinitis, primary biliary cirrhosis, irritable bowel disease and Sjogren syndrome.

54. The use according to any of claims 44-48, wherein said disorder is selected from myasthenia gravis, rheumatoid arthritis, multiple sclerosis, transplant rejection and graft vs. host disease.
55. A diagnostic composition including the composition of any of claims 1-38.
56. A diagnostic composition including the composition of any of claims 1-38 and a cross-linking moiety or moieties.
57. A method for killing a cell expressing an antigen on the surface of said cell comprising the step of treating the cell with a plurality of antigen-binding domains of any one of claims 1-38, wherein said antibody-based antigen-binding domains are part of a multivalent polypeptide, and where neither cytotoxic entities nor immunological mechanisms are needed to causes or leads to said killing
58. A method to identify patients that can be treated with a composition of any of claims 1-38, formulated in a pharmaceutically acceptable carrier and/or diluent comprising the steps of
  - a. Isolating cells from a patient;
  - b. Contacting said cells with the composition of any of claims 1-38; and
  - c. Measuring the degree of killing or immunosuppression of said cells.
59. A kit to identify patients that can be treated with a composition of any of claims 1-38, formulated in a pharmaceutically acceptable carrier and/or diluent comprising
  - a. A composition of any of claims 1-38; and
  - b. Means to measure the degree of killing or immunosuppression of said cells.
60. A kit comprising
  - a. a composition according to any one of claims 1-38, and
  - b. a cross-linking moiety.
61. A kit comprising
  - a. a composition according to any one of claims 1-38, and
  - b. a detectable moiety or moieties, and
  - c. reagents and/or solutions to effect and/or detect binding of (i) to an antigen.
62. A cytotoxic composition comprising a composition of any one of claims 1-38 operably linked to a cytotoxic agent.



63. An immunogenic composition comprising a composition of any one of claims 1-38 operably linked to an immunogenic agent.
64. A method to kill a cell comprising contacting said cell with a composition of any one of claims 1-38 operably linked a cytotoxic or immunogenic agent.
65. The use of a composition of any one of claims 1-38 operable linked a cytotoxic or immunogenic agent for preparing a pharmaceutical preparation for the treatment of animals.
66. A composition including a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for a human MHC class II antigen with a  $K_d$  of  $1\mu\text{M}$  or less, wherein treating cells expressing said antigen with said polypeptide causes or leads to suppression of an immune response.
67. A composition including a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for human HLA DR antigen, wherein treating cells expressing HLA DR with said polypeptide causes or leads to suppression of an immune response, and wherein said antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.
68. A composition including a polypeptide comprising at least one antibody-based antigen-binding domain with a binding specificity for a human MHC class II antigen with a  $K_d$  of  $1\mu\text{M}$  or less, said antigen-binding domain being isolated by a method which includes isolation of VL and VH domains of human composition from a recombinant antibody library by ability to bind to human MHC class II antigen, wherein treating cells expressing MHC Class II with said polypeptide causes or leads to suppression of an immune response.
69. The composition of claim 68, wherein the method for isolating the antigen-binding domain includes the further steps of:
  - a. generating a library of variants at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
  - b. isolation of VL and VH domains from the library of variants by ability to bind to human MHC class II antigen with a  $K_d$  of  $1\mu\text{M}$  or less;

- c. (optionally) repeating steps (a) and (b) with at least one other of the CDR1, CDR2 and CDR3 sequences.
70. The composition of any of claims 67, 68 or 69, wherein said antigen-binding domain binds to HLA-DR
71. The composition of any of claims 66 or 70 wherein said antigen-binding domain binds to the  $\beta$ -chain of HLA-DR.
72. The composition of claim 71, wherein said antigen-binding domain binds to an epitope of the first domain of the  $\beta$ -chain of HLA-DR.
73. The composition of any of claims 66-72, wherein said cells are lymphoids cells.
74. The composition of any of claims 66-72, wherein said cells are non-lymphoid cells and express MHC class II antigens.
75. The composition of any of claims 66-74, having an  $IC_{50}$  for suppressing an immune response of 1  $\mu$  M or less.
76. The composition of any of claims 66-74, having an  $IC_{50}$  for inhibition of IL-2 secretion of 1  $\mu$  M or less
77. The composition of any of claims 66-74, having an  $IC_{50}$  for inhibiting T cell proliferation of 1  $\mu$  M or less
78. The composition of any of claims 66-77, wherein said antigen-binding domain binds to one or more HLA-DR types selected from the group consisting of DR1-0101, DR2-15021, DR3-0301, DR4Dw4-0401, DR4Dw10-0402, DR4Dw14-0404, DR6-1302, DR6-1401, DR8-8031, DR9-9012, DRw53-B4\*0101 and DRw52-B3\*0101.
79. The composition of claim 78, wherein said antigen-binding domain binds to at least 5 different of said HLA-DR types.
80. The composition of any of claims 66-79, wherein said antigen-binding domain includes a combination of a VH domain and a VL domain, wherein said combination is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-6, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

81. The composition of any one of claims 66-77, wherein said antigen-binding domain includes of a combination of HuCAL VH2 and HuCAL Vλ1, wherein the VH CDR3, VL CDR1 And VL CDR3 is found in one of the clones taken from the list of MS-GPC-1, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

82. The composition of any of claims 66-77, wherein said antigen-binding domain includes a combination of HuCAL VH2 and HuCAL Vλ1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

wherein each n independently represents any amino acid residue; and/or

wherein the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

wherein each n independently represents any amino acid residue.

83. The composition of claim 82, wherein the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.

84. The composition of any of claims 66-77, wherein said antigen-binding domain competes for antigen binding with an antibody including a combination of HuCAL VH2 and HuCAL Vλ1, wherein the VH CDR3 sequence is taken from the consensus CDR3 sequence

nnnnRGnFDn

each n independently represents any amino acid residue; and/or

the VL CDR3 sequence is taken from the consensus CDR3 sequence

QSYDnnnn

each n independently represents any amino acid residue.

85. The composition of claim 84, wherein the VH CDR3 sequence is SPRYGAFDY and/or the VL CDR3 sequence is QSYDLIRH or QSYDMNVH.

86. The composition of any of claims 66-85, wherein said antigen-binding domain includes a VL CDR1 sequence represented in the general formula

SGSnnNIGnNYVn

wherein each n independently represents any amino acid residue.

87. The composition of claim 86, wherein the CDR1 sequence is SGSESNIGNNYVQ.
88. The composition of any one of claims 66-85, wherein said suppression of an immune response is brought about by or manifests itself in down-regulation of expression of said antigen expressed on the surface of said cell.
89. The composition of any one of claims 66-85, wherein said suppression of an immune response is brought about by or manifests itself in inhibition of the interaction between said cell and other cells, wherein said interaction would normally lead to an immune response.
90. The composition of any one of claims 66-85, wherein said suppression of the immune response is brought about by or manifests itself in the killing of said cells.
91. The composition of claim 90, wherein said killing is mediated by binding of a plurality of antigen-binding domains, wherein said antibody-based antigen-binding domains are part of a multivalent polypeptide, and where neither cytotoxic entities nor immunological mechanisms are needed to causes or leads to said killing.
92. The composition of any one of claims 66-91, formulated in a pharmaceutically acceptable carrier and/or diluent
93. A pharmaceutical preparation comprising the composition of claim 75 in an amount sufficient to suppress an immune response in an animal.
94. A pharmaceutical preparation comprising the composition of claim 76 in an amount sufficient to inhibit IL-2 secretion in an animal.
95. A pharmaceutical preparation comprising the composition of claim 77 in an amount sufficient to inhibit T cell proliferation in an animal.
96. The use of a composition of any one of claims 66-91, for preparing a pharmaceutical preparation for the treatment of animals, such as where said animals are human.
97. A nucleic acid including a protein coding sequence for a polypeptide of the composition of any of claims 66-91.
98. A vector comprising the coding sequence of claim 97, and a transcriptional regulatory sequence operably linked thereto.
99. A host cell harboring a nucleic acid of claim 97 or the vector of claim 98.
100. A method for the production of an immunosuppressive composition, comprising culturing the cells of claim 99 under conditions wherein the nucleic acid is expressed.

101. A method for suppressing activation of a cell of the immune system, such as expressing HLA DR, comprising treating the cell with a composition of any of claims 66-92.
102. A method for suppressing proliferation of a cell of the immune system, such as expressing HLA DR, comprising treating the cell with a composition of any of claims 66-92.
103. A method for suppressing IL-2 secretion by a cell of the immune system, such as expressing HLA DR, comprising treating the cell with a composition of any of claims 66-92.
104. A method for immunosuppressing a patient, comprising administering to the patient an effective amount of a composition of any of claims 66-92 to reduce the level of immunological responsiveness in the patient.
105. A method for killing a cell expressing an antigen on the surface of said cell comprising the step of treating the cell with a plurality of antigen-binding domains of any one of claims 66-87, wherein said antibody-based antigen-binding domains are part of a multivalent polypeptide, and where neither cytotoxic entities nor immunological mechanisms are needed to causes or leads to said killing, such where said antigen is HLA DR.
106. The use according to claim 96, wherein said treatment is the treatment of a disorder selected from rheumatoid arthritis, juvenile arthritis, multiple sclerosis, Grave's disease, insulin-dependent diabetes, narcolepsy, psoriasis, systemic lupus erythematosus, ankylosing spondylitis, transplant rejection, graft vs. host disease, Hashimoto's disease, myasthenia gravis, pemphigus vulgaris, glomerulonephritis, thyroiditis, pancreatitis, insulinitis, primary biliary cirrhosis, irritable bowel disease and Sjogren syndrome.
107. The use according to claim 96, wherein said treatment is the treatment of a disorder selected from myasthenia gravis, rheumatoid arthritis, multiple sclerosis, transplant rejection and graft vs. host disease.
108. A method of suppressing the interaction of a cell of the immune system with an other cell, comprising contacting the cell with the composition of any of claims 66-92.
109. A method for conducting a pharmaceutical business comprising:
  - (i) isolating one or more antigen-binding domains that bind to antigens expressed on the surface of human cells;

- (ii) generating a multivalent composition, such as multivalent polypeptide, comprising a plurality of said antigen-binding domains, which multivalent composition kills with an  $EC_{50}$  of 50nM or less transformed or activated cells that express said antigen, where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing.;
- (iii) conducting therapeutic profiling of the multivalent composition, for efficacy and toxicity in animals;
- (iv) preparing a package insert describing the multivalent composition for treatment of proliferative disorders; and
- (v) marketing the multivalent composition for treatment of proliferative disorders.

110. A method for conducting a life science business comprising:

- (i) isolating one or more antigen-binding domains that bind to antigens expressed on the surface of human cells;
- (ii) generating a multivalent composition, such as multivalent polypeptide, comprising a plurality of said antigen-binding domains, which multivalent composition kills with an  $EC_{50}$  of 50nM or less transformed or activated cells expressing said antigen where neither cytotoxic entities nor immunological mechanisms are needed to cause or lead to said killing;
- (iii) licensing, jointly developing or selling, to a third party, the rights for selling the multivalent composition.

111. The method of any of claims 109 or 110, wherein the antigen-binding domain is isolated by a method which includes

- a. isolation of VL and VH domains of human composition from a recombinant antibody library by ability to bind to HLA DR,
- b. generating a library of variants at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
- c. isolation of VL and VH domains from the library of variants by ability bind to HLA DR with a  $K_d$  of 1 $\mu$ M or less.

112. A method for conducting a pharmaceutical business comprising:

- (i) isolating one or more antigen-binding domains that bind to MHC class II expressed on the surface of human cells with a  $K_d$  of 1 $\mu$ M or less;
  - (ii) generating a composition comprising said antigen-binding domains, which composition is immunosuppressant with an  $IC_{50}$  of 100nM or less;
  - (iii) conducting therapeutic profiling of the composition for efficacy and toxicity in animals;
  - (iv) preparing a package insert describing the use of the composition for immunosuppression therapy; and
  - (v) marketing the composition for use as an immunosuppressant.
113. A method for conducting a life science business comprising:
- (i) isolating one or more antigen-binding domains that bind to MHC class II expressed on the surface of human cells with a  $K_d$  of 1 $\mu$ M or less;
  - (ii) generating a composition comprising said antigen-binding domains, which composition is immunosuppressant with an  $IC_{50}$  of 100nM or less;
  - (iii) licensing, jointly developing or selling, to a third party, the rights for selling the composition.
114. The method of any of claims 112 or 113, wherein the antigen-binding domain is isolated by a method which includes
- a. isolation of VL and VH domains of human composition from a recombinant antibody library by ability to bind to HLA DR,
  - b. generating a library of variants at least one of the CDR1, CDR2 and CDR3 sequences of one or both of the VL and VH domains, and
  - c. isolation of VL and VH domains from the library of variants by ability to bind to HLA DR with a  $K_d$  of 1 $\mu$ M or less.
115. The method of any of claims 109-114, wherein said antigen-binding domain comprises a combination of VH and VL domains found in the clones taken from the list of MS-GPC-1, MS-GPC-8, MS-GPC-10, MS-GPC-8-1, MS-GPC-8-6, MS-GPC-8-9, MS-GPC-8-10, MS-GPC-8-17, MS-GPC-8-18, MS-GPC-8-27, MS-GPC-8-6-2, MS-GPC-8-6-19, MS-GPC-8-6-27, MS-GPC-8-6-45, MS-GPC-8-6-13, MS-GPC-8-6-47, MS-GPC-8-10-57, MS-GPC-8-27-7, MS-GPC-8-27-10 and MS-GPC-8-27-41.

**Figure 1a**

	MS-GPC- 8-27-7	MS-GPC- 8-27-10	MS-GPC- 8-6-13	MS-GPC- 8-27-41	MS-GPC- 8-6-47	MS-GPC- 8-10-57	MS-GPC- 8-6-27	MS-GPC- 8	MS-GPC- 8-6
Plastic	-0,004	-0,02	-0,022	-0,025	-0,001	0,005	0,007	-0,022	-0,018
BSA	-0,003	-0,019	-0,021	-0,022	0,008	0,003	0,003	-0,016	-0,019
Testosterone -BSA	-0,005	-0,01	-0,012	-0,007	0,011	0,003	0,002	-0,009	-0,012
Lysozyme	-0,005	-0,079	-0,079	-0,073	0,013	0,014	0,006	-0,081	-0,072
human Apotransferrin	-0,009	-0,016	-0,018	-0,018	-0,005	-0,008	-0,004	-0,014	-0,016
MHCII (DRA*0101/ DRB1*0401)	1,549	1,493	1,467	1,525	1,4	1,256	1,297	1,058	1,306



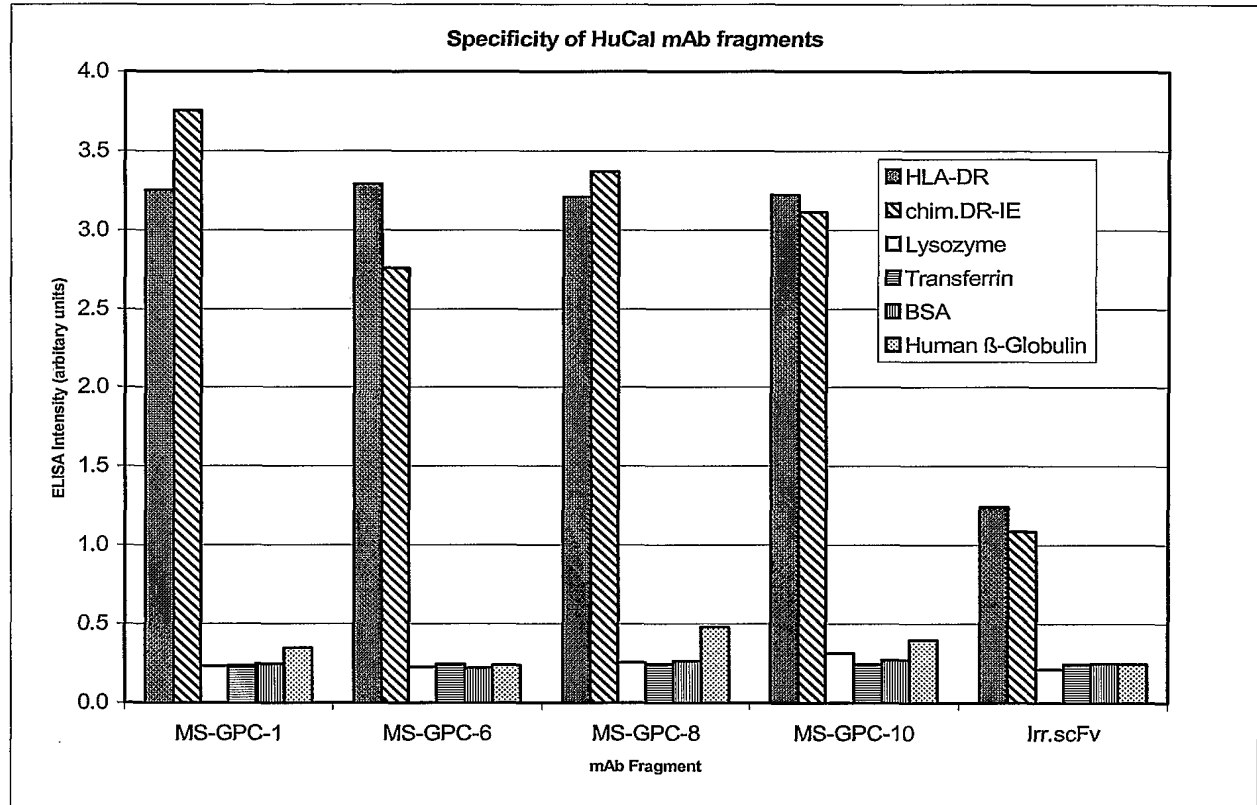
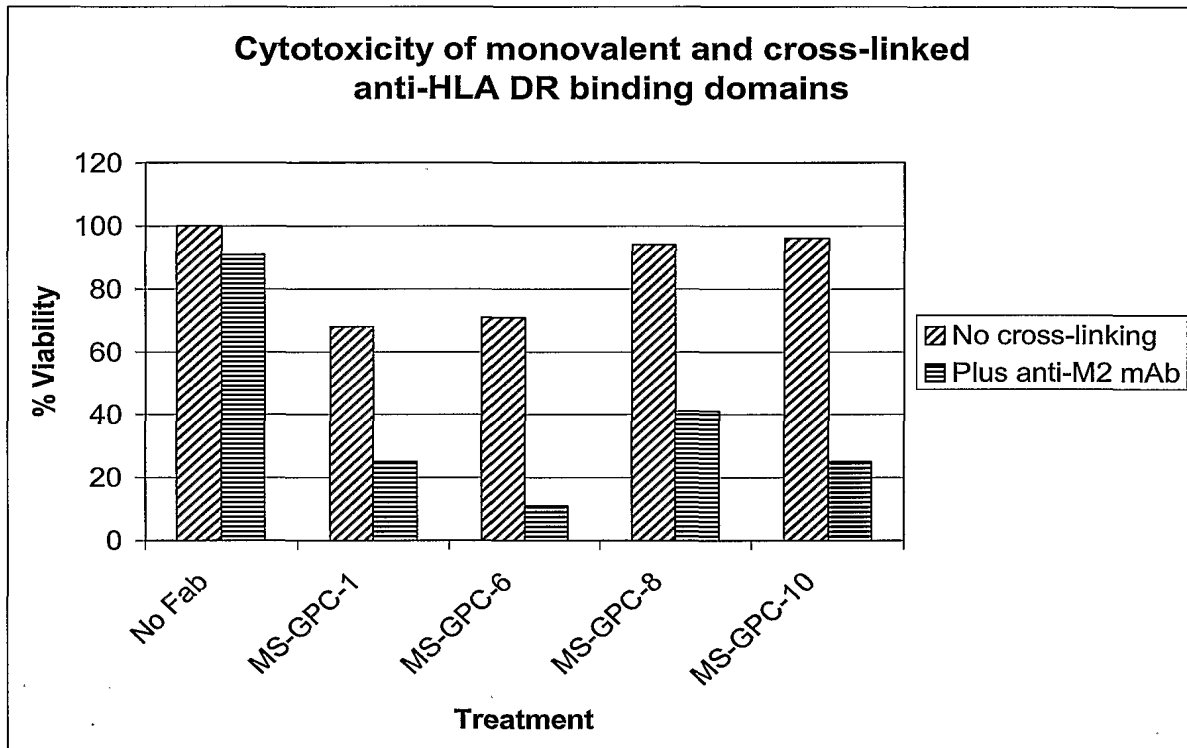
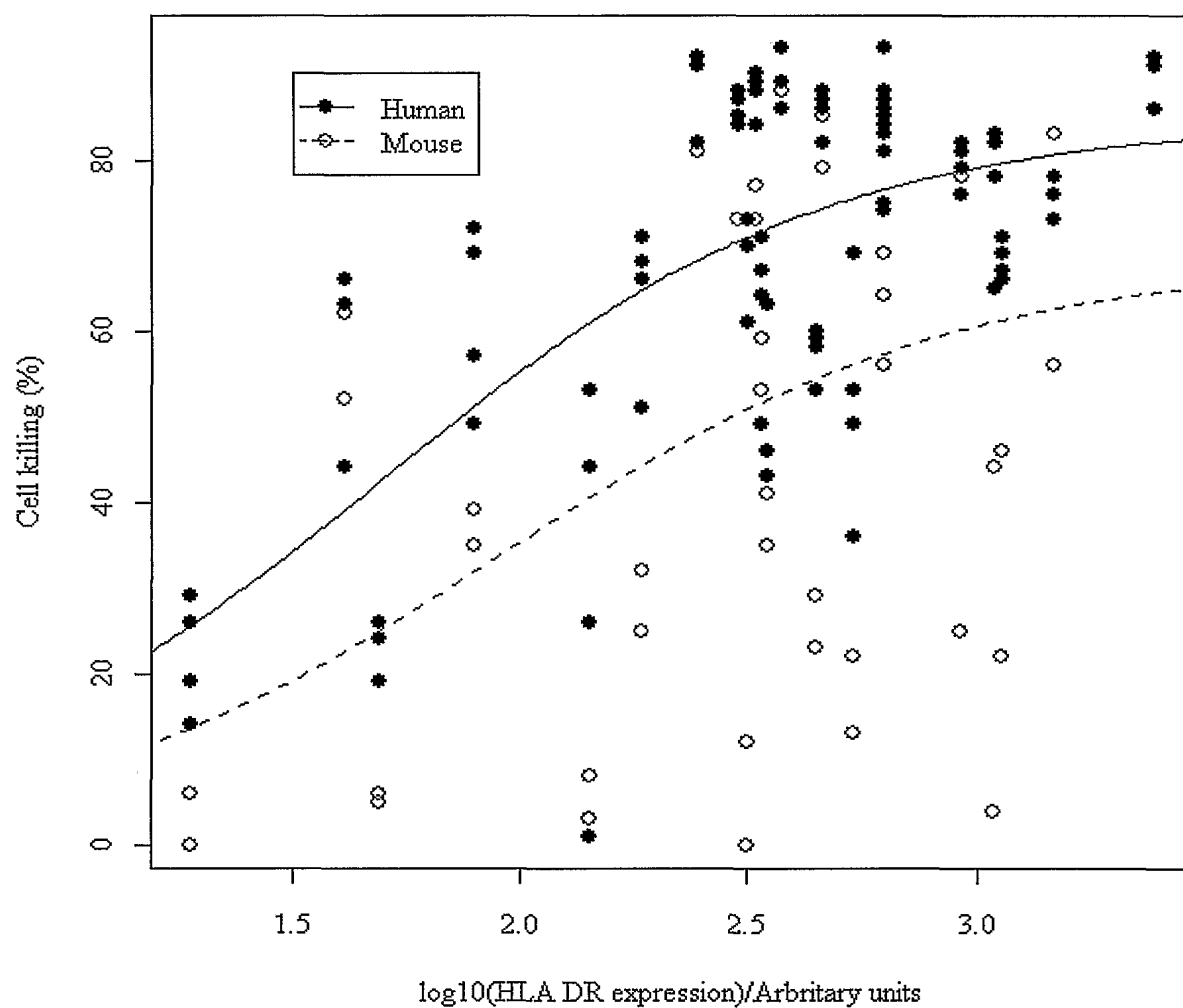
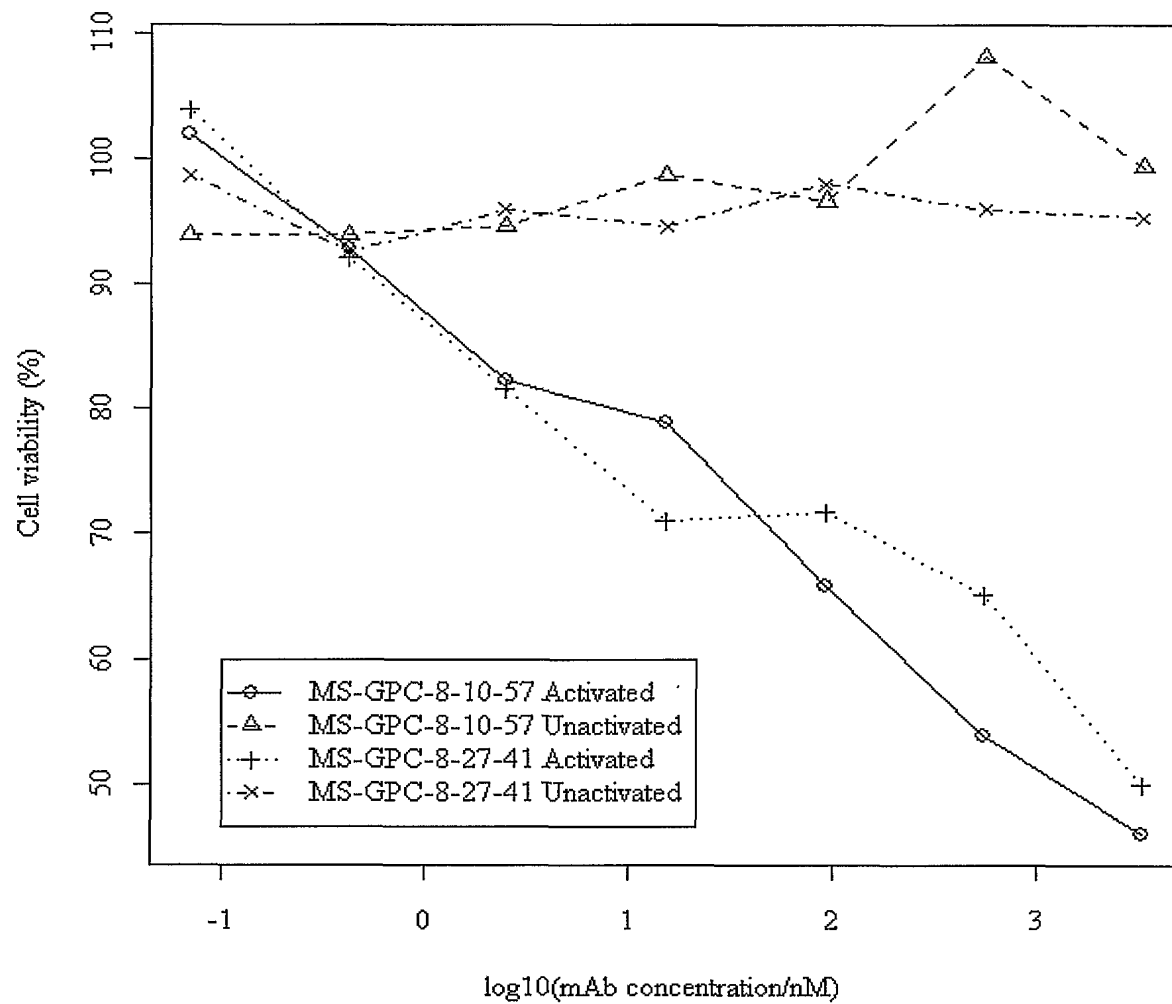
**Figure 1b**

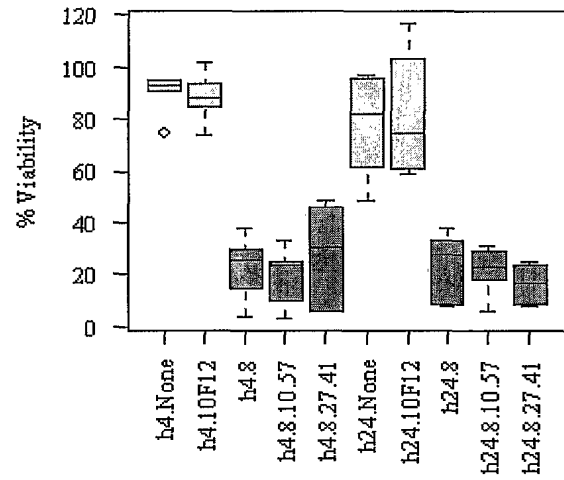
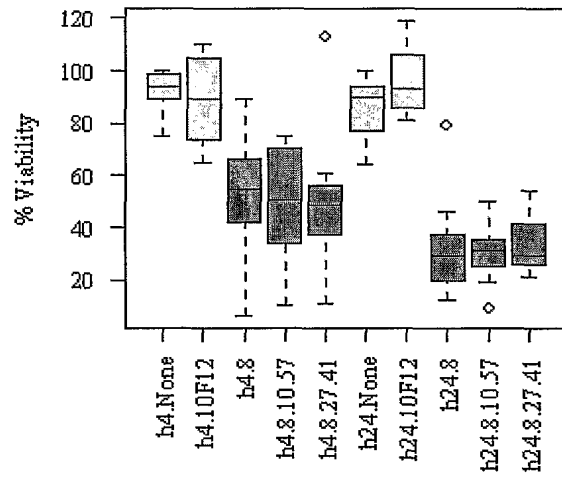
Figure 2

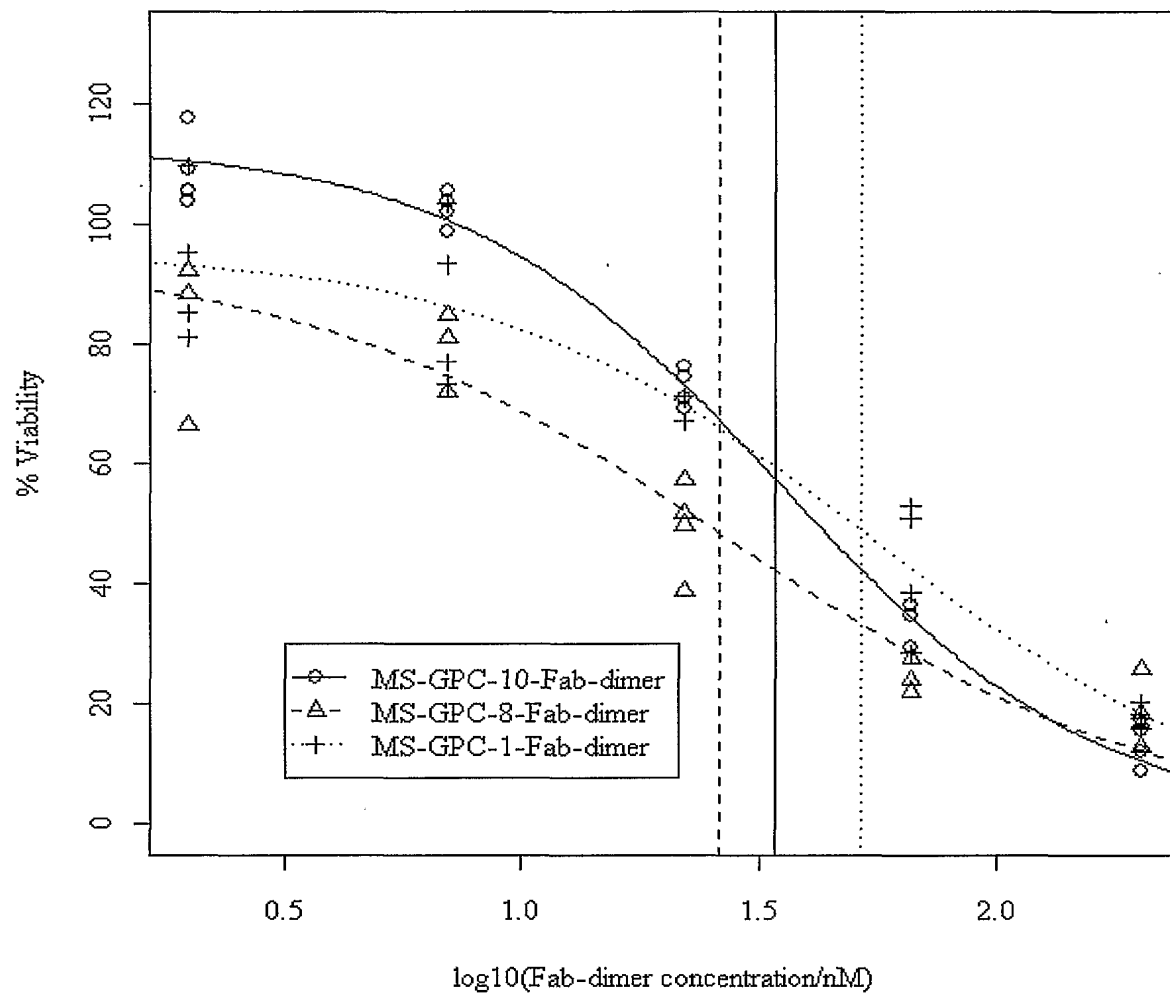
Cell line	HLA-	DRB1*	scFv				IgG			
			MS-GPC-1	MS-GPC-6	MS-GPC-8	MS-GPC-10	MS-GPC-8	MS-GPC-8-10-57	MS-GPC-8-27-41	MS-GPC-8-6-13
LG2	DR1	0101	+	+	+	+	+	+	+	+
E4181324	DR2	15021	+	+	+	+	+	+	+	+
VAVY	DR3	0301	+	+	+	+	+	+	+	+
Priess	DR4Dw4	0401	+	+	+	+	+	+	+	+
TS10	DR4Dw10	0402	+	+	+	+	+	+	+	+
BIN40	DR4Dw14	0404	+	+	+	+	+	+	+	+
WT47	DR6	1302	+	+	+	+	+	+	+	+
TEM	DR6	1401	+	+	+	+	+	+	+	+
TAB089	DR8	8031	+	+	+	+	+	+	+	+
DKB	DR9	9012	+	+	+	+	+	+	+	+
L257.6	DRw53	B4*0101	+/-	+	+	+/-	nt	nt	nt	nt
L105.1	DRw52	B3*0101	+	+	+	+	nt	nt	nt	nt
L25.4	DPw4/w4.2	DP0103/0402	-	+	-	-	nt	nt	nt	nt
L256.12	DPw2/w2.1	DP0202/0201	-	+/-	-	-	nt	nt	nt	nt
L21.3	DQ7/w2	DQ0201/0602	-	+	+	-	nt	nt	nt	nt

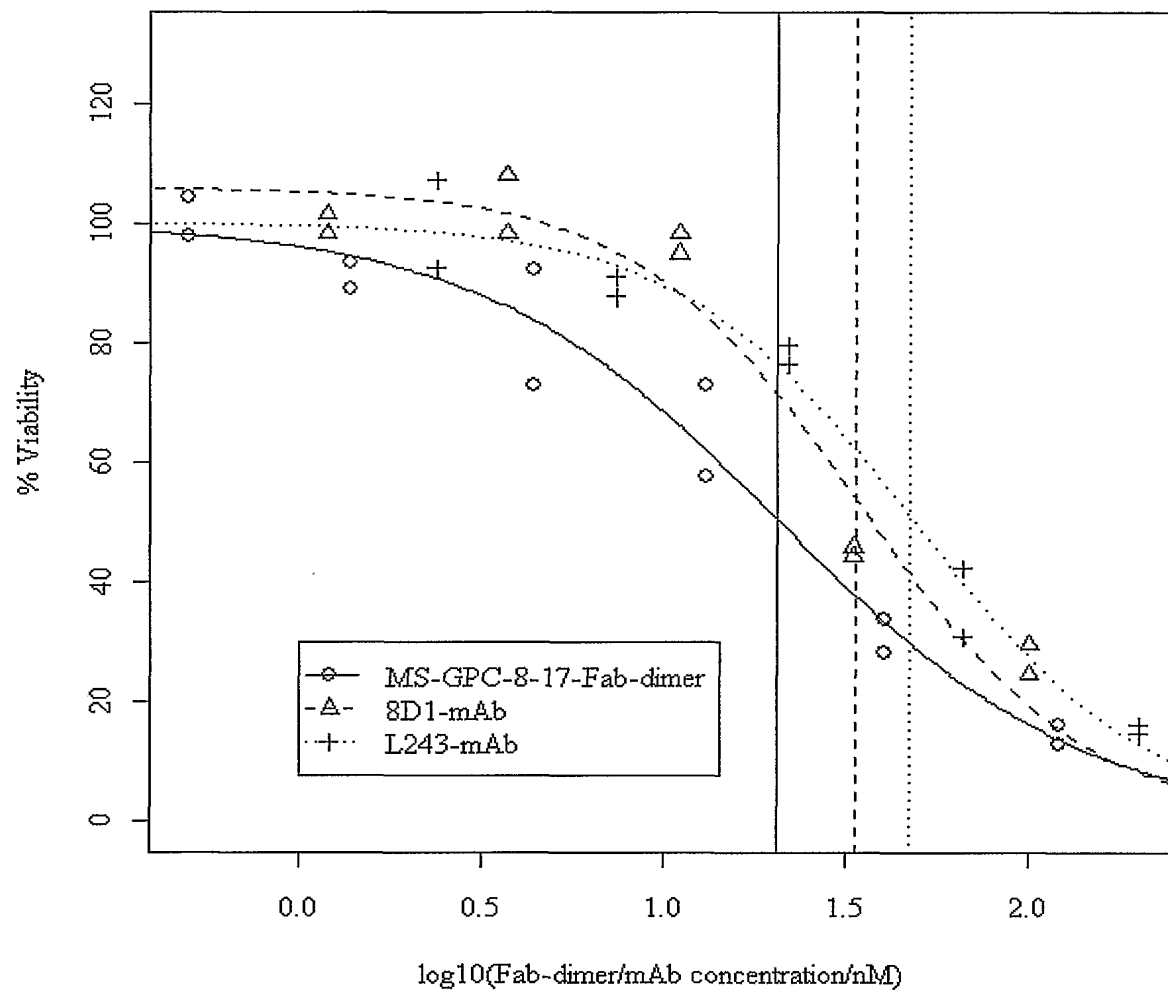
**Figure 3**

**Figure 4**

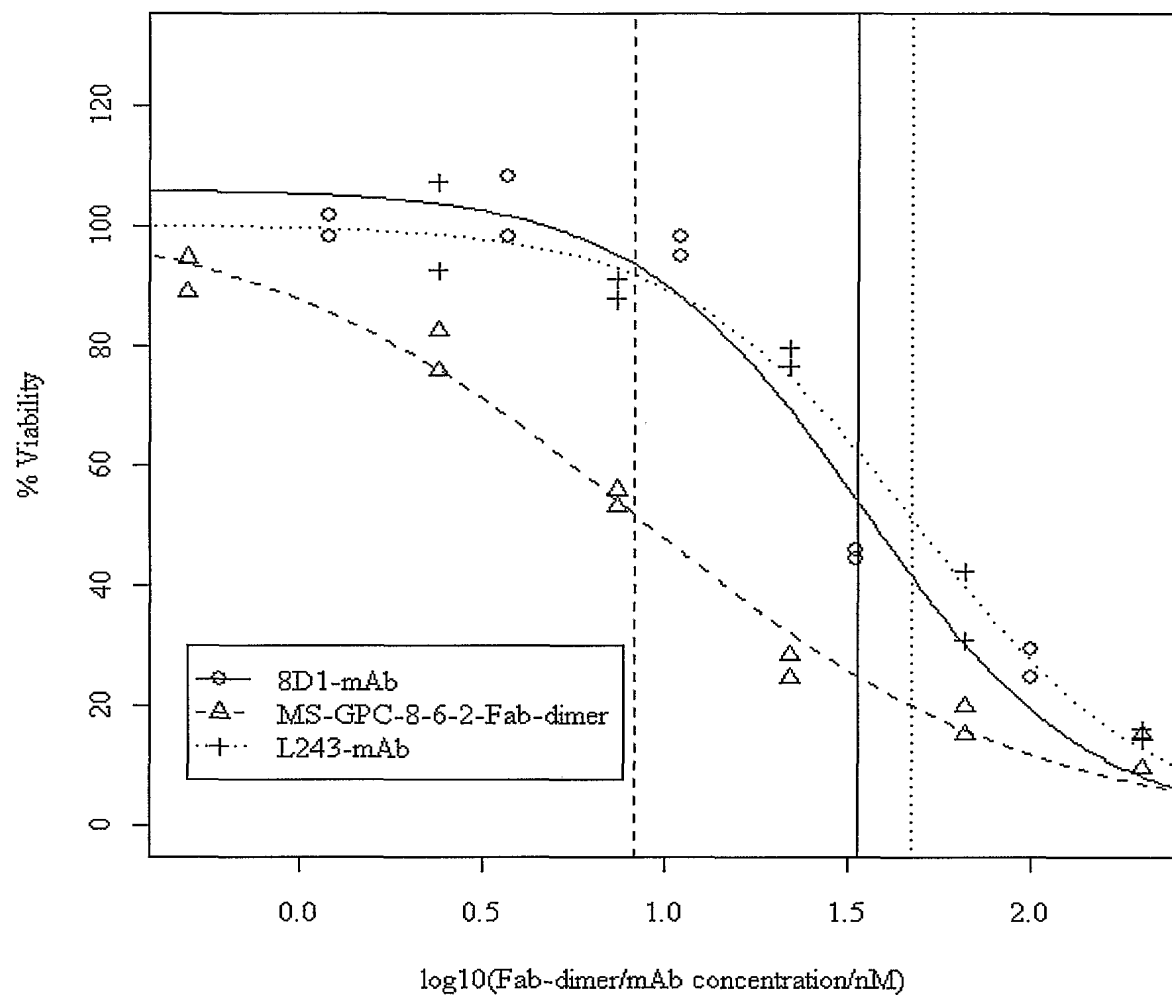
**Figure 5**

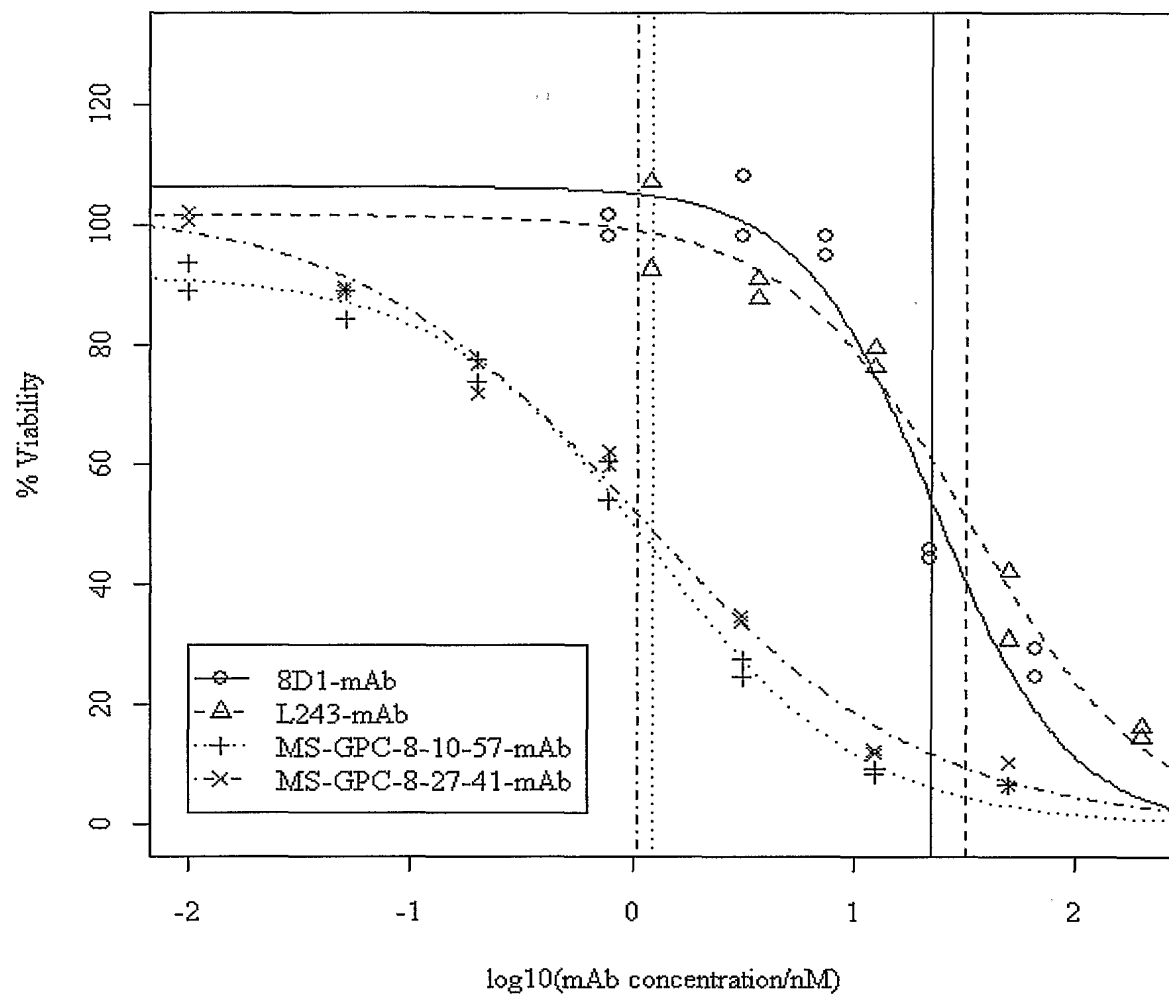
**Figure 6**

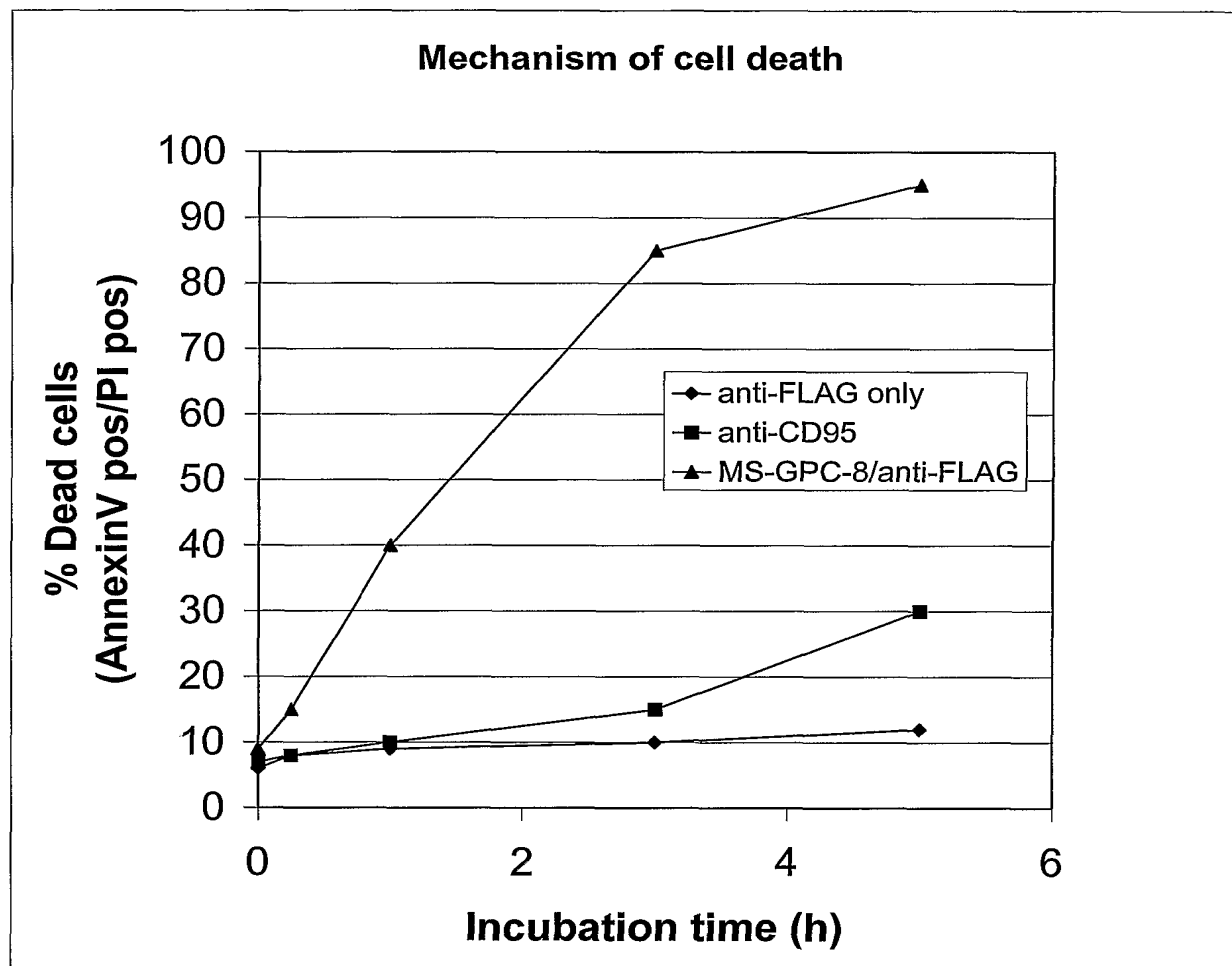
**Figure 7a**

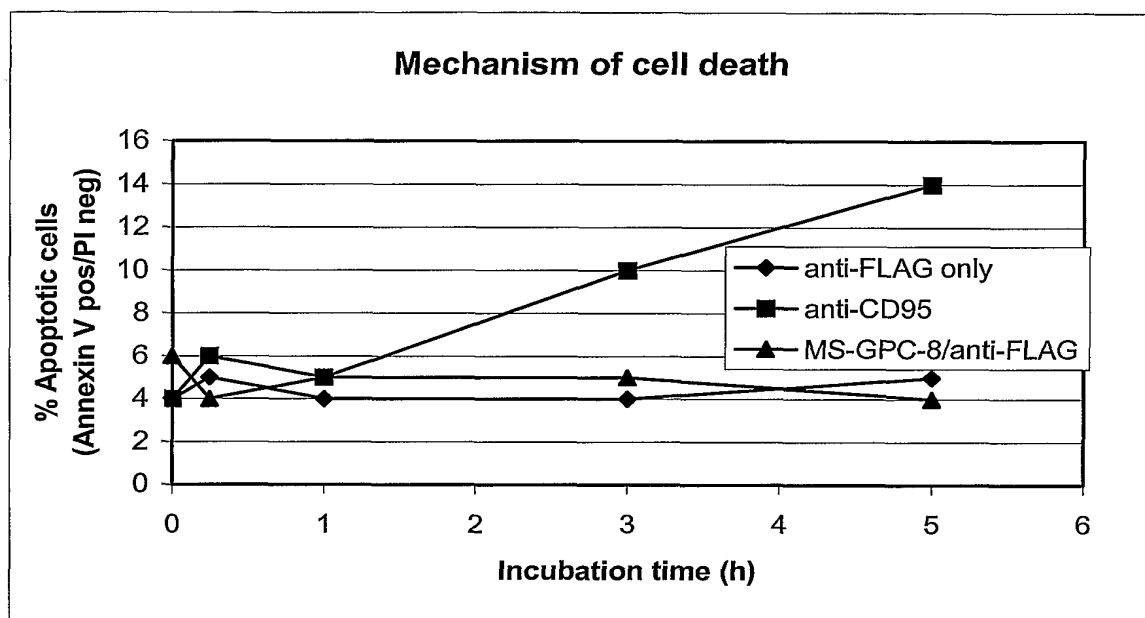
**Figure 7b**

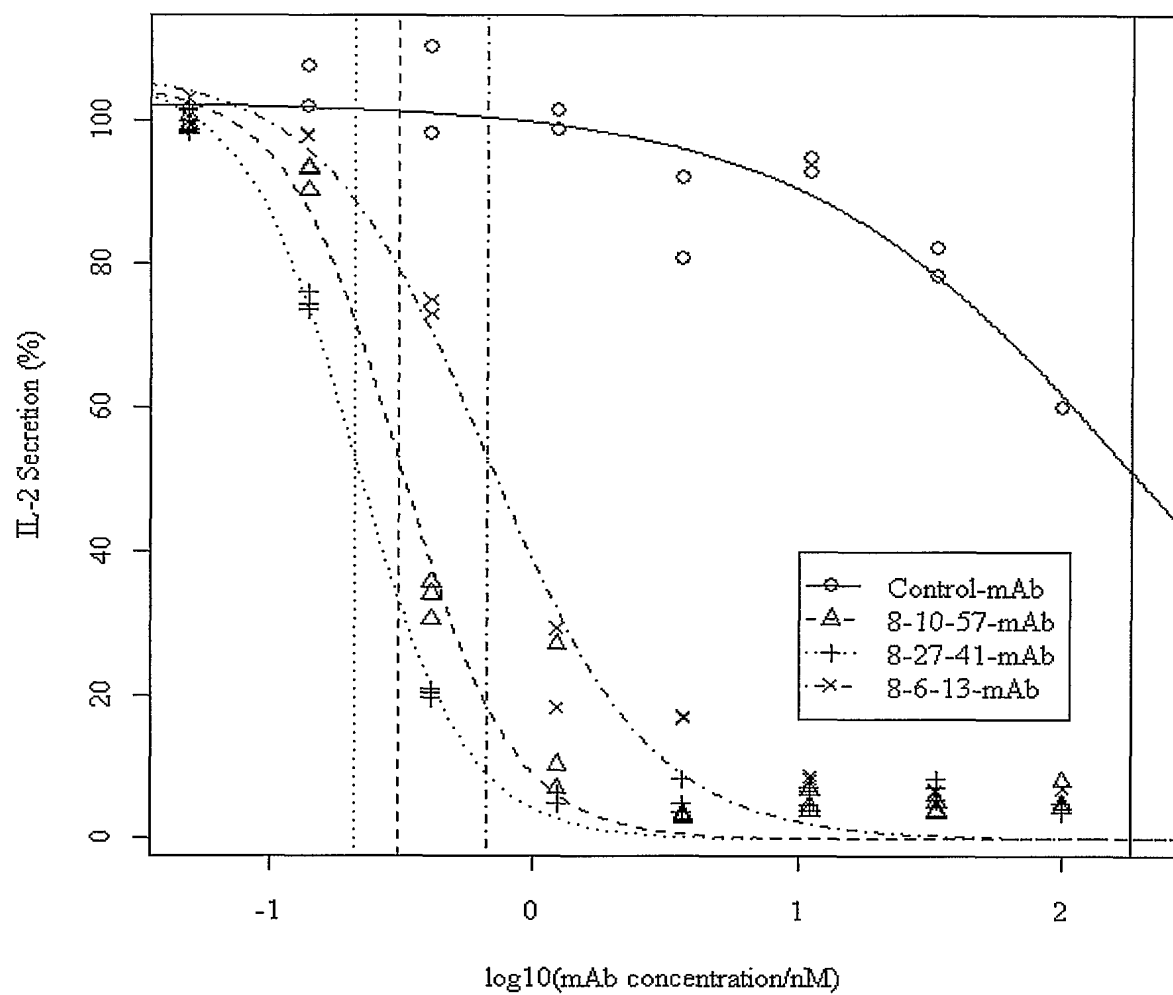


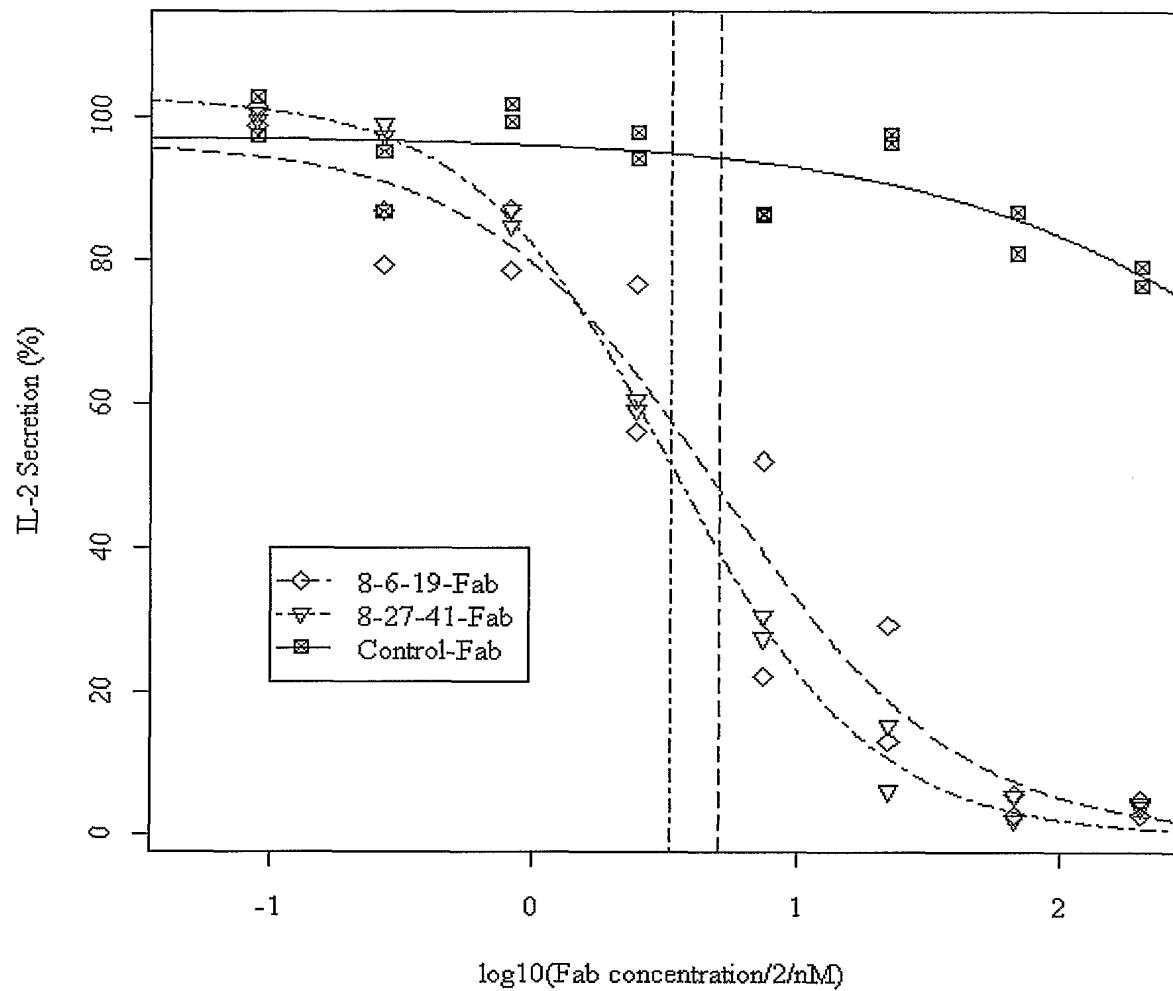
**Figure 7c**

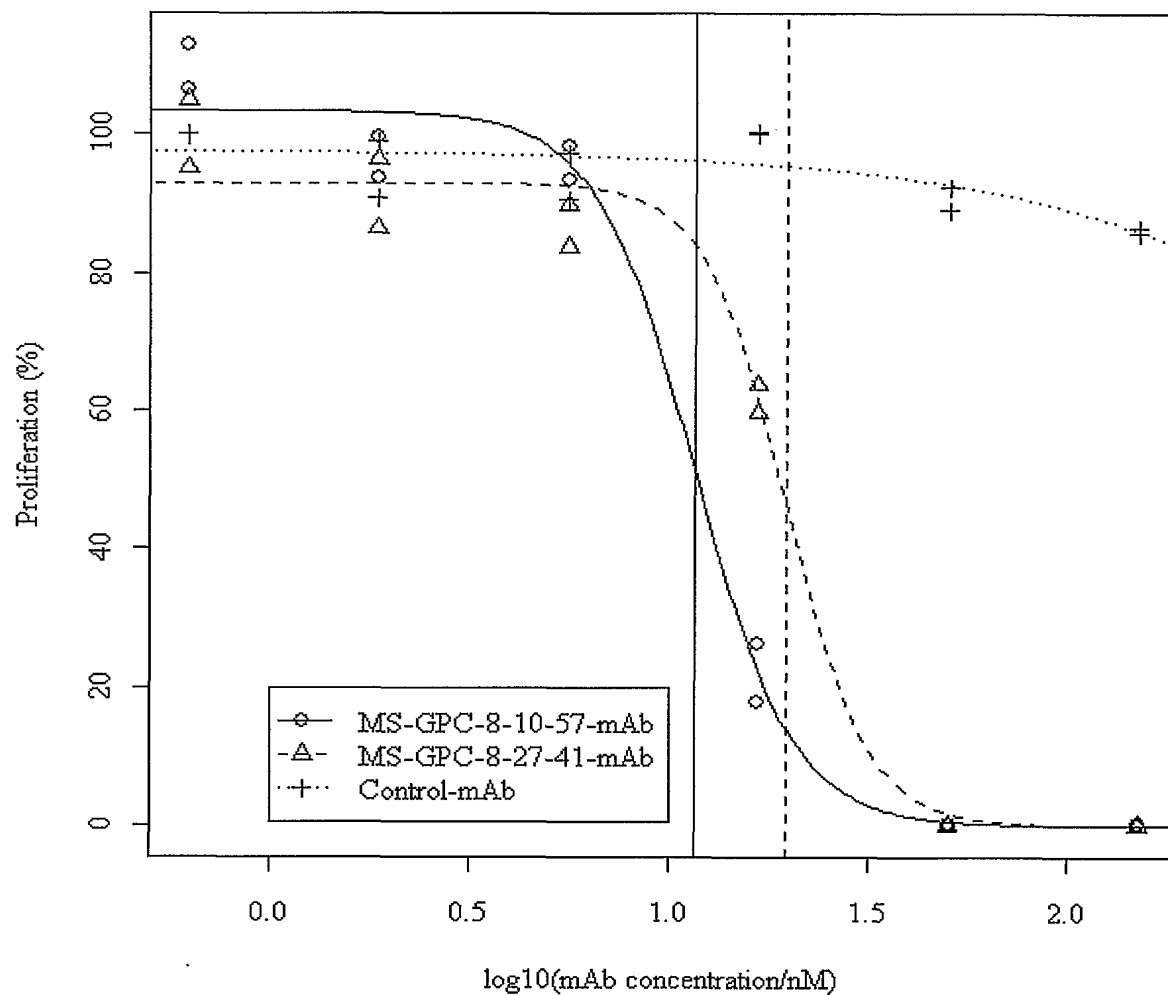
**Figure 7d**

**Figure 8a**

**Figure 8b**

**Figure 9a**

**Figure 9b**

**Figure 10**





# Figure 11 (cont)

XbaISphI

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1 AGAGCATGCG TAGGAGAAAA TAAATGAAA CAAAGCACTA TTGCACTGGC  
TCTCGTACGC ATCCTCTTTT ATTTTACTTT GTTTCGTGAT AACGTGACCG

51 ACTCTTACCG TTGCTCTTCA CCCCTGTTAC CAAAGCCGAC TACAAAGATG  
TGAGAATGGC AACGAGAAGT GGGGACAATG GTTTCGGCTG ATGTTTCTAC

MfeI

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101 AAGTGCAATT GGTTCAGTCT GGCGCGGAAG TGAAAAAACC GGGCAGCAGC  
TTCACGTTAA CCAAGTCAGA CCGCGCCTTC ACTTTTTTGG CCCGTCGTCG

BspEI

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151 GTGAAAGTGA GCTGCAAAGC CTCCGGAGGC ACTTTTAGCA GCTATGCGAT  
CACTTTCCT CACGCTTTCG GAGGCCTCCG TGAAAATCGT CGATACGCTA

XhoI

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AvaI

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201 TAGCTGGGTG CGCCAAGCCC CTGGGCAGGG TCTCGAGTGG ATGGGCGGCA  
ATCGACCCAC GCGGTTCGGG GACCCGTCCC AGAGCTCACC TACCCGCCGT

BstEII

~

251 TTATTCCGAT TTTTGGCACG GCGAACTACG CGCAGAAGTT TCAGGGCCGG  
AATAAGGCTA AAAACCGTGC CGCTTGATGC GCGTCTTCAA AGTCCCGGCC

BstEII

~~~~~

301 GTGACCATTA CCGCGGATGA AAGCACCAGC ACCGCGTATA TGGAAGTGA  
CACTGGTAAT GGCGCCTACT TTCGTGGTGC TGGCGCATAT ACCTTGACTC

EagI

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BssHII

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351 CAGCCTGCGT AGCGAAGATA CGGCCGTGTA TTATTGCGCG CGTTATTATG  
GTCGGACGCA TCGCTTCTAT GCCGGCACAT AATAACGCGC GCAATAATAC

StyI

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401 ATCGTATGTA TAATATGGAT TATTGGGGCC AAGGCACCCT GGTGACGGTT  
TAGCATACAT ATTATACCTA ATAACCCCGG TTCCGTGGGA CCACTGCCAA

BlpI

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CelII

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451 AGCTCAGCGG GTGGCGGTTT TGGCGGCGGT GGGAGCGGTG GCGGTGGTTC  
TCGAGTCGCC CACCGCCAAG ACCGCCGCCA CCCTCGCCAC CGCCACCAAG

EcoRV

~~~~~

501 TGGCGGTGGT GGTTCGATA TCGAACTGAC CCAGCCGCCT TCAGTGAGCG

ACCGCCACCA CCAAGGCTAT AGCTTGA CTG GGTCTGGCGGA AGTCACTCGC

SexAI

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551 TTGCACCAGG TCAGACCGCG CGTATCTCGT GTAGCGGCGA TGCCTGGGC  
AACGTGGTCC AGTCTGGCGC GCATAGAGCA CATCGCCGCT ACGCGACCCG

XmaI

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KpnI

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Acc65I

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SmaI

~~~~~

AvaI

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601 GATAAATACG CGAGCTGGTA CCAGCAGAAA CCCGGGCAGG CGCCAGTTCT  
CTATTTATGC GCTCGACCAT GGTCGTCTTT GGGCCCGTCC GCGGTCAAGA

Bsu36I

~~~~~

651 GGTGATTTAT GATGATTCTG ACCGTCCCTC AGGCATCCCG GAACGCTTTA  
CCACTAAATA CTAATAAGAC TGGCAGGGAG TCCGTAGGGC CTTGCGAAAT

BamHI

~~~~~

701 GCGGATCCAA CAGCGGCAAC ACCGCGACCC TGACCATTAG CGGCACTCAG  
CGCCTAGGTT GTCGCCGTTG TGGCGCTGGG ACTGGTAATC GCCGTGAGTC

BpuAI

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BbsI

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751 GCGGAAGACG AAGCGGATTA TTATTGCCAG AGCTATGACG CTCATATGCG  
CGCCTTCTGC TTCGCCTAAT AATAACGGTC TCGATACTGC GAGTATACGC

HpaI

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MscI

~~~~~

EcoRI

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801 TCCTGTGTTT GGCGGCGGCA CGAAGTTAAC CGTTCTTGGC CAGGAATTCG  
AGGACACAAA CCGCCGCCGT GCTTCAATTG GCAAGAACCG GTCCTTAAGC

851 AGCAGAAGCT GATCTCTGAG GAGGATCTGA ACTAGGGTGG TGGCTCTGGT  
TCGTCTTCGA CTAGAGACTC CTCCTAGACT TGATCCCACC ACCGAGACCA

901 TCCGGTGATT TTGATTATGA AAAGATGGCA AACGCTAATA AGGGGGCTAT  
AGGCCACTAA AACTAATACT TTTCTACCGT TTGCGATTAT TCCCCGATA

gIIIseq9 100.0%

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951 GACCGAAAAT GCCGATGAAA ACGCGCTACA GTCTGACGCT AAAGGCAAAC  
CTGGCTTTTA CGGCTACTTT TGCGCGATGT CAGACTGCGA TTTCCGTTTG

ClaI

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1001 TTGATTCTGT CGCTACTGAT TACGGTGCTG CTATCGATGG TTTCATTGGT  
AACTAAGACA GCGATGACTA ATGCCACGAC GATAGCTACC AAAGTAACCA

1051 GACGTTTCCG GCCTTGCTAA TGGTAATGGT GCTACTGGTG ATTTTGCTGG  
CTGCAAAGGC CGGAACGATT ACCATTACCA CGATGACCAC TAAAACGACC

20/49

1101	CTCTAATTCC GAGATTAAGG	CAAATGGCTC GTTTACCGAG	AAGTCGGTGA TTCAGCCACT	CGGTGATAAT GCCACTATTA	TCACCTTTAA AGTGGA AATT
1151	TGAATAATTT ACTTATTAAA	CCGTCAATAT GGCAGTTATA	TTACCTTCCC AATGGAAGGG	TCCCTCAATC AGGGAGTTAG	GGTTGAATGT CCAAC TTACA
1201	CGCCCTTTTG GCGGGAAAAC	TCTTTGGCGC AGAAACCGCG	TGGTAAACCA ACCATT TGGT	TATGAATTTT ATACTTAAAA	CTATTGATTG GATAACTAAC
1251	TGACAAAATA ACTGTTTTAT	AAC TTATTCC TTGAATAAGG	GTGGTGTCTT CACCACAGAA	TGCGTTTCTT ACGCAAAGAA	TTATATGTTG AATATACAAC
1301	CCACCTTTAT GGTGGAAATA	GTATGTATTT CATACATAAA	TCTACGTTTG AGATGCAAAC	CTAACATACT GATTGTATGA	GCGTAATAAG CGCATTATTC
HindIII ~~~~~					
1351	GAGTCTTGAT CTCAGAACTA	AAGCTTGACC TTCGAACTGG	TGTGAAGTGA ACACTTCACT	AAAATGGCGC TTTTACCGCG	AGATTGTGCG TCTAACACGC
OGIII3 100.0% =====					
1401	ACATTTTTTT TGTA AAAAAA	TGTCTGCCGT ACAGACGGCA	TTAATGAAAT AATTACTTTA	TGTAAACGTT ACATTTGCAA	AATATTTTGT TTATAAAACA
1451	TAAAATTCGC ATTTTAAAGCG	GTTAAATTTT CAATTTAAAA	TGTTAAATCA ACAATTTAGT	GCTCATTTTT CGAGTAAAAA	TAACCAATAG ATTGGTTATC
1501	GCCGAAATCG CGGCTTTAGC	GCAAAATCCC CGTTTTTAGG	TTATAAATCA AATATTTAGT	AAAGAATAGA TTTCTTATCT	CCGAGATAGG GGCTCTATCC
1551	GTTGAGTGTT CAACTCACAA	GTTCCAGTTT CAAGGTCAAA	GGAACAAGAG CCTTGTTCTC	TCCACTATTA AGGTGATAAT	AAGAACGTGG TTCTTGCAAC
1601	ACTCCAACGT TGAGGTTGCA	CAAAGGGCGA GTTTCCCGCT	AAAACCGTCT TTT TGGCAGA	ATCAGGGCGA TAGTCCCGCT	TGGCCCACTA ACCGGGTGAT
1651	CGAGAACCAT GCTCTTG GTA	CACCCTAATC GTGGGATTAG	AAGTTTTTTG TTCAAAAAAC	GGGTCGAGGT CCCAGCTCCA	GCCGTAAAGC CGGCATTTCG
1701	ACTAAATCGG TGATTTAGCC	AACCCTAAAG TTGGGATTTC	GGAGCCCCCG CCTCGGGGGC	ATTTAGAGCT TAAATCTCGA	TGACGGGGAA ACTGCCCTT
1751	AGCCGGCGAA TCGGCCGCTT	CGTGGCGAGA GCACCGCTCT	AAGGAAGGGA TTCCTTCCCT	AGAAAGCGAA TCTTT CGCTT	AGGAGCGGGC TCCTCGCCCC
1801	GCTAGGGCGC CGATCCCGCG	TGGCAAGTGT ACCGTT CACA	AGCGGTCACG TCGCCAGTGC	CTGCGCGTAA GACGCGCATT	CCACCACACC GGTGGTGTGG
1851	CGCCGCGCTT GCGGCGCGAA	AATGCGCCGC TTACGCGGCG	TACAGGGCGC ATGTCCCGCG	GTGCTAGCCA CACGATCGGT	TGTGAGCAAA ACACTCGTTT
1901	AGGCCAGCAA TCCGGTCGTT	AAGGCCAGGA TTCCGGTCCT	ACCGTAAAAA TGGCATT TTT	GGCCGCGTTG CCGGCGCAAC	CTGGCGTTTT GACCGCAAAA
1951	TCCATAGGCT AGGTATCCGA	CCGCCCCCCT GGCGGGGGGA	GACGAGCATC CTGCTCGTAG	ACAAAAATCG TGTTTTTAGC	ACGCTCAAGT TGCGAGTTCA

2001 CAGAGGTGGC GAAACCCGAC AGGACTATAA AGATACCAGG CGTTTCCCCC  
 GTCTCCACCG CTTTGGGCTG TCCTGATATT TCTATGGTCC GCAAAGGGGG  
 2051 TGGAAGCTCC CTCGTGCGCT CTCCTGTTCC GACCCTGCCG CTTACCGGAT  
 ACCTTCGAGG GAGCACGCGA GAGGACAAGG CTGGGACGGC GAATGGCCTA  
 2101 ACCTGTCCGC CTTTCTCCCT TCGGGAAGCG TGGCGCTTTC TCATAGCTCA  
 TGGACAGGCG GAAAGAGGGA AGCCCTTCGC ACCGCGAAAG AGTATCGAGT  
 2151 CGCTGTAGGT ATCTCAGTTC GGTGTAGGTC GTTCGCTCCA AGCTGGGCTG  
 GCGACATCCA TAGAGTCAAG CCACATCCAG CAAGCGAGGT TCGACCCGAC

## ApaLI

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2201 TGTGCACGAA CCCCCGTTT AGTCCGACCG CTGCGCCTTA TCCGGTAACT  
 ACACGTGCTT GGGGGGCAAG TCAGGCTGGC GACGCGGAAT AGGCCATTGA  
 2251 ATCGTCTTGA GTCCAACCCG GTAAGACACG ACTTATCGCC ACTGGCAGCA  
 TAGCAGAACT CAGGTTGGGC CATTCTGTGC TGAATAGCGG TGACCGTTCGT  
 2301 GCCACTGGTA ACAGGATTAG CAGAGCGAGG TATGTAGGCG GTGCTACAGA  
 CGGTGACCAT TGTCTAATC GTCTCGCTCC ATACATCCGC CACGATGTCT  
 2351 GTTCTTGAAG TGGTGGCCTA ACTACGGCTA CACTAGAAGA ACAGTATTTG  
 CAAGAACTTC ACCACCGGAT TGATGCCGAT GTGATCTTCT TGTCATAAAC  
 2401 GTATCTGCGC TCTGCTGTAG CCAGTTACCT TCGGAAAAAG AGTTGGTAGC  
 CATAGACGCG AGACGACATC GGTCAATGGA AGCCTTTTTT TCAACCATCG  
 2451 TCTTGATCCG GCAAACAAAC CACCGCTGGT AGCGGTGGTT TTTTGTGTTG  
 AGAACTAGGC CGTTTGTTTG GTGGCGACCA TCGCCACCAA AAAACAAAC  
 2501 CAAGCAGCAG ATTACGCGCA GAAAAAAGG ATCTCAAGAA GATCCTTTGA  
 GTTCGTCGTC TAATGCGCGT CTTTTTTTCC TAGAGTTCTT CTAGGAAACT  
 2551 TCTTTTCTAC GGGGTCTGAC GCTCAGTGGA ACGAAAACTC ACGTTAAGGG  
 AGAAAAGATG CCCAGACTG CGAGTCACCT TGCTTTTGAG TGCAATTCCC  
 2601 ATTTTGGTCA GATCTAGCAC CAGGCGTTTA AGGGCACCAA TAACTGCCTT  
 TAAAACCAGT CTAGATCGTG GTCCGCAAAT TCCCGTGGTT ATTGACGGAA  
 2651 AAAAAAATTA CGCCCCGCC TGCCACTCAT CGCAGTACTG TTGTAATTCA  
 TTTTTTTAAT GCGGGGCGGG ACGGTGAGTA GCGTCATGAC AACATTAAGT  
 2701 TTAAGCATT C TGCCGACATG GAAGCCATCA CAAACGGCAT GATGAACCTG  
 AATTCGTAAG ACGGCTGTAC CTTCGGTAGT GTTTGCCGTA CTACTTGGAC  
 2751 AATCGCCAGC GGCATCAGCA CCTTGTCGCC TTGCGTATAA TATTTGCCCA  
 TTAGCGGTCTG CCGTAGTCGT GGAACAGCGG AACGCATATT ATAAACGGGT  
 2801 TAGTGAAAAC GGGGGCGAAG AAGTTGTCCA TATTGGCTAC GTTTAAATCA  
 ATCACTTTTG CCCCCGCTTC TTCAACAGGT ATAACCGATG CAAATTTAGT  
 2851 AAACTGGTGA AACTCACCCA GGGATTGGCT GAGACGAAAA ACATATTCTC  
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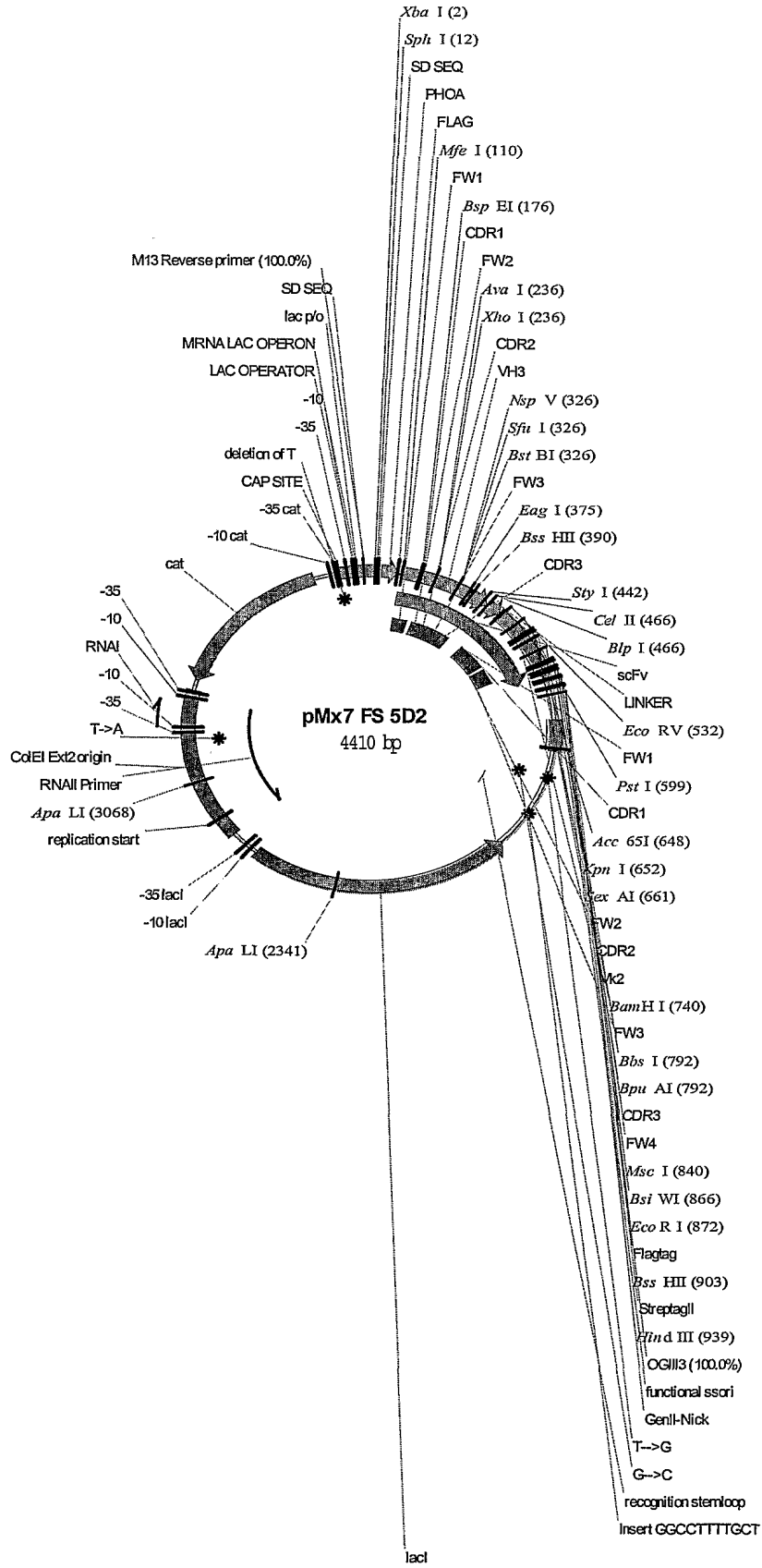
22/49

|                           |                           |                          |                          |                          |                           |
|---------------------------|---------------------------|--------------------------|--------------------------|--------------------------|---------------------------|
| 2901                      | AATAAACCCCT<br>TTATTTGGGA | TTAGGGAAAT<br>AATCCCTTTA | AGGCCAGGTT<br>TCCGGTCCAA | TTCACCGTAA<br>AAGTGGCATT | CACGCCACAT<br>GTGCGGTGTA  |
| 2951                      | CTTGCGAATA<br>GAACGCTTAT  | TATGTGTAGA<br>ATACACATCT | AACTGCCGGA<br>TTGACGGCCT | AATCGTCGTG<br>TTAGCAGCAC | GTATTCACCTC<br>CATAAGTGAG |
| 3001                      | CAGAGCGATG<br>GTCTCGCTAC  | AAAACGTTTC<br>TTTTGCAAAG | AGTTTGCTCA<br>TCAAACGAGT | TGGAAAACGG<br>ACCTTTTGCC | TGTAACAAGG<br>ACATTGTTCC  |
| 3051                      | GTGAACACTA<br>CACTTGATGAT | TCCCATATCA<br>AGGGTATAGT | CCAGCTCACC<br>GGTCGAGTGG | GTCTTTCATT<br>CAGAAAGTAA | GCCATACGGA<br>CGGTATGCCT  |
| 3101                      | ACTCCGGGTG<br>TGAGGCCAC   | AGCATTCATC<br>TCGTAAGTAG | AGGCGGGCAA<br>TCCGCCGTT  | GAATGTGAAT<br>CTTACACTTA | AAAGGCCGGA<br>TTTCCGGCCT  |
| 3151                      | TAAAACTTGT<br>ATTTTGAACA  | GCTTATTTTT<br>CGAATAAAAA | CTTTACGGTC<br>GAAATGCCAG | TTTAAAAAGG<br>AAATTTTTC  | CCGTAATATC<br>GGCATTATAG  |
| 3201                      | CAGCTGAACG<br>GTCGACTTGC  | GTCTGGTTAT<br>CAGACCAATA | AGGTACATTG<br>TCCATGTAAC | AGCAACTGAC<br>TCGTTGACTG | TGAAATGCCT<br>ACTTTACGGA  |
| 3251                      | CAAAATGTTC<br>GTTTTACAAG  | TTTACGATGC<br>AAATGCTACG | CATTGGGATA<br>GTAACCCTAT | TATCAACGGT<br>ATAGTTGCCA | GGTATATCCA<br>CCATATAGGT  |
| 3301                      | GTGATTTTTT<br>CACTAAAAAA  | TCTCCATTTT<br>AGAGGTAAAA | AGCTTCCTTA<br>TCGAAGGAAT | GCTCCTGAAA<br>CGAGGACTTT | ATCTCGATAA<br>TAGAGCTATT  |
| 3351                      | CTCAAAAAAT<br>GAGTTTTTTA  | ACGCCCCGTA<br>TGCGGGCCAT | GTGATCTTAT<br>CACTAGAATA | TTCATTATGG<br>AAGTAATACC | TGAAAGTTGG<br>ACTTTCAACC  |
| 3401                      | AACCTCACCC<br>TTGGAGTGGG  | GACGTCTAAT<br>CTGCAGATTA | GTGAGTTAGC<br>CACTCAATCG | TCACTCATT<br>AGTGAGTAAT  | GGCACCCAG<br>CCGTGGGGTC   |
| 3451                      | GCTTTACACT<br>CGAAATGTGA  | TTATGCTTCC<br>AATACGAAGG | GGCTCGTATG<br>CCGAGCATAC | TTGTGTGGAA<br>AACACACCTT | TTGTGAGCGG<br>AACACTCGCC  |
| M13 Reverse primer 100.0% |                           |                          |                          |                          |                           |
| =====                     |                           |                          |                          |                          |                           |
| 3501                      | ATAACAATTT<br>TATTGTTAAA  | CACACAGGAA<br>GTGTGTCCTT | ACAGCTATGA<br>TGTCGATACT | CCATGATTAC<br>GGTACTAATG | GAATTTCT<br>CTTAAAGA      |

XbaI

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Figure 12



## Figure 12 (cont)

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      XbaI   SphI
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1    TCTAGAGCAT GCGTAGGAGA AAATAAAATG AAACAAAGCA CTATTGCACT
    AGATCTCGTA CGCATCCTCT TTTATTTTAC TTTGTTTCGT GATAACGTGA

51   GGCACCTCTTA CCGTTGCTCT TCACCCCTGT TACCAAAGCC GACTACAAAG
    CCGTGAGAAT  GGCAACGAGA AGTGGGGACA ATGGTTTCGG CTGATGTTTC

      MfeI
      ~~~~~
101  ATGAAGTGCA ATTGGTGGA  AGCGGCGGCG GCCTGGTGCA ACCGGGCGGC
    TACTTCACGT TAACCACCTT TCGCCGCCGC CGGACCACGT TGGCCCGCCG

      BspEI
      ~~~~~
151  AGCCTGCGTC TGAGCTGCGC GGCCTCCGGA TTTACCTTTA GCAGCTATGC
    TCGGACGCAG ACTCGACGCG CCGGAGGCCT AAATGGAAAT CGTCGATACG

      XhoI
      ~~~~~
      AvaI
      ~~~~~
201  GATGAGCTGG GTGCGCCAAG CCCCTGGGAA GGGTCTCGAG TGGGTGAGCG
    CTACTCGACC CACGCGGTTC GGGGACCCTT CCCAGAGCTC ACCCACTCGC

251  CGATTAGCGG TAGCGGCGGC AGCACCTATT ATGCGGATAG CGTGAAAGGC
    GCTAATCGCC ATCGCCGCCG TCGTGGATAA TACGCCTATC GCACTTTCCG

      BstBI
      ~~~~~
      SfuI
      ~~~~~
      NspV
      ~~~~~
301  CGTTTTACCA TTTCACGTGA TAATTCGAAA AACACCCTGT ATCTGCAAAT
    GCAAATGGT  AAAGTGCAC TTAAGCTTT TTGTGGGACA TAGACGTTTA

      EagI           BssHII
      ~~~~~           ~~~~~
351  GAACAGCCTG CGTGCGGAAG ATACGGCCGT GTATTATTGC GCGCGTGTTA
    CTTGTCGGAC  GCACGCCTTC TATGCCGGCA CATAATAACG CGCGCACAAT

      StyI
      ~~~~~
401  AGAAGCATTT TTCTCGTAAG AATTGGTTTG ATTATTGGGG CCAAGGCACC
    TCTTCGTAAA  AAGAGCATTC TTAACCAAAC TAATAACCCC GGTTC CGTGG
  
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                BlpI
                ~~~~~~
                CelII
                ~~~~~~
451  CTGGTGACGG TTAGCTCAGC GGGTGGCGGT TCTGGCGGCG GTGGGAGCGG
    GACCACTGCC AATCGAGTCG CCCACCGCCA AGACCGCCGC CACCCTCGCC

                                EcoRV
                                ~~~~~~
501  TGGCGGTGGT TCTGGCGGTG GTGGTTCCGA TATCGTGATG ACCCAGAGCC
    ACCGCCACCA AGACCGCCAC CACCAAGGCT ATAGCACTAC TGGGTCTCGG

  PstI
                                                ~~~~~~
551  CACTGAGCCT GCCAGTGACT CCGGGCGAGC CTGCGAGCAT TAGCTGCAGA
    GTGACTCGGA CGGTCACTGA GGCCCGCTCG GACGCTCGTA ATCGACGTCT

                                                KpnI
                                                ~~~~~~
  Acc65I
                                                ~~~~~~
601  AGCAGCCAAA GCCTGCTGCA TAGCAACGGC TATAACTATC TGGATTGGTA
    TCGTCGGTTT CGGACGACGT ATCGTTGCCG ATATTGATAG ACCTAACCAT

KpnI
~~
Acc65I      SexAI
~~          ~~~~~~
651  CCTTCAAAAA CCAGGTCAAA GCCCGCAGCT ATTAATTTAT CTGGGCAGCA
    GGAAGTTTTT GGTCCAGTTT CGGGCGTCGA TAATTAAATA GACCCGTCGT

                                                BamHI
                                                ~~~~~~
701  ACCGTGCCAG TGGGGTCCCG GATCGTTTTA GCGGCTCTGG ATCCGGCACC
    TGGCACGGTC ACCCCAGGGC CTAGCAAAAT CGCCGAGACC TAGGCCGTGG

  BpuAI
                                                ~~~~~~
                                                BbsI
                                                ~~~~~~
751  GATTTTACCC TGAAAATTAG CCGTGTGGAA GCTGAAGACG TGGGCGTGTA
    CTAAAATGGG ACTTTTAATC GGCACACCTT CGACTTCTGC ACCCGCACAT

  MscI
                                                ~~~~~~
801  TTATTGCCAG CAGCATTATA CCACCCCGCC GACCTTTGGC CAGGGTACGA
    AATAACGGTC GTCGTAATAT GGTGGGGCGG CTGGAAACCG GTCCCATGCT

```



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                                BsiWI   EcoRI
                                ~~~~~
851  AAGTTGAAAT TAAACGTACG GAATTCGACT ATAAAGATGA CGATGACAAA
    TTCAACTTTA ATTTGCATGC CTTAAGCTGA TATTTCTACT GCTACTGTTT

    BssHII                               HindIII
    ~~~~~                               ~~~~~
901  GGCGCGCCGT GGAGCCACCC GCAGTTTGA  AAATGATAAG CTTGACCTGT
    CCGCGCGGCA CCTCGGTGGG CGTCAAACCT TTTACTATTC GAACTGGACA
                                           OGIII3 100.0%
                                           =====

951  GAAGTGAAAA ATGGCGCAGA TTGTGCGACA TTTTTTTTGT CTGCCGTTTA
    CTTCACTTTT TACCGCGTCT AACACGCTGT AAAAAAACA GACGGCAAAT
    OGIII3 100.0%
    =====

1001 ATTAAAGGGG GGGGGGGGCC GGCCTGGGGG GGGGTGTACA TGAAATTGTA
    TAATTTCCCC CCCCCCCCGG CCGGACCCCC CCCACATGT ACTTTAACAT

1051 AACGTTAATA TTTTGTTAAA ATTCGCGTTA AATTTTGTGT AAATCAGCTC
    TTGCAATTAT AAAACAATTT TAAGCGCAAT TTAAAAACAA TTTAGTCGAG

1101 ATTTTTTAAC CAATAGGCCG AAATCGGCAA AATCCCTTAT AAATCAAAAG
    TAAAAAATTG GTTATCCGGC TTTAGCCGTT TTAGGAATA TTTAGTTTTC

1151 AATAGACCGA GATAGGGTTG AGTGTTGTTC CAGTTTGGAA CAAGAGTCCA
    TTATCTGGCT CTATCCCAAC TCACAACAAG GTCAAACCTT GTTCTCAGGT

1201 CTATTAAAGA ACGTGGACTC CAACGTCAAA GGGCGAAAAA CCGTCTATCA
    GATAATTTCT TGCACCTGAG GTTGCAGTTT CCCGCTTTTT GGCAGATAGT

1251 GGGCGATGGC CCACTACGAG AACCATCACC CTAATCAAGT TTTTGGGGT
    CCCGCTACCG GGTGATGCTC TTGGTAGTGG GATTAGTTCA AAAAACCCCA

1301 CGAGGTGCCG TAAAGCACTA AATCGGAACC CTAAAGGGAG CCCCCGATTT
    GCTCCACGGC ATTTTCGTGAT TTAGCCTTGG GATTTCCCTC GGGGGCTAAA

1351 AGAGCTTGAC GGGGAAAGCC GGCGAACGTG GCGAGAAAGG AAGGGAAGAA
    TCTCGAACTG CCCCTTTCGG CCGCTTGAC  CGTCTTTCC TTCCCTTCTT

1401 AGCGAAAGGA GCGGGCGCTA GGGCGCTGGC AAGTGTAGCG GTCACGCTGC
    TCGCTTTCCT CGCCGCGGAT CCCGCGACCG TTCACATCGC CAGTGCGACG

1451 GCGTAACCAC CACACCCGCC GCGCTTAATG CGCCGCTACA GGGCGCGTGC
    CGCATTGGTG GTGTGGGCGG CGCGAATTAC GCGGCGATGT CCCGCGCACG
```

|      |            |            |            |             |            |
|------|------------|------------|------------|-------------|------------|
| 1501 | TAGACTAGTG | TTTAAACCGG | ACCGGGGGGG | GGCTTAAGTG  | GGCTGCAAAA |
|      | ATCTGATCAC | AAATTTGGCC | TGGCCCCCCC | CCGAATTCAC  | CCGACGTTTT |
| 1551 | CAAAACGGCC | TCCTGTCAGG | AAGCCGCTTT | TATCGGGTAG  | CCTCACTGCC |
|      | GTTTTGCCGG | AGGACAGTCC | TTCGGCGAAA | ATAGCCCATC  | GGAGTGACGG |
| 1601 | CGCTTTCCAG | TCGGGAAACC | TGTCGTGCCA | GCTGCATCAG  | TGAATCGGCC |
|      | GCGAAAGGTC | AGCCCTTTGG | ACAGCACGGT | CGACGTAGTC  | ACTTAGCCGG |
| 1651 | AACGCGCGGG | GAGAGGCGGT | TTGCGTATTG | GGAGCCAGGG  | TGGTTTTTCT |
|      | TTGCGCGCCC | CTCTCCGCCA | AACGCATAAC | CCTCGGTCCC  | ACCAAAAAGA |
| 1701 | TTTCACCAGT | GAGACGGGCA | ACAGCTGATT | GCCCTTCACC  | GCCTGGCCCT |
|      | AAAGTGGTCA | CTCTGCCCCG | TGTCGACTAA | CGGGAAGTGG  | CGGACCGGGA |
| 1751 | GAGAGAGTTG | CAGCAAGCGG | TCCACGCTGG | TTTGCCCCAG  | CAGGCGAAAA |
|      | CTCTCTCAAC | GTCGTTCGCC | AGGTGCGACC | AAACGGGGTC  | GTCCGCTTTT |
| 1801 | TCCTGTTTGA | TGGTGGTCAG | CGGCGGGATA | TAACATGAGC  | TGTCCTCGGT |
|      | AGGACAAACT | ACCACCAGTC | GCCGCCCTAT | ATTGTACTCG  | ACAGGAGCCA |
| 1851 | ATCGTCGTAT | CCCACTACCG | AGATGTCCGC | ACCAACGCGC  | AGCCCGGACT |
|      | TAGCAGCATA | GGGTGATGGC | TCTACAGGCG | TGGTTGCGCG  | TCGGGCCTGA |
| 1901 | CGGTAATGGC | ACGCATTGCG | CCCAGCGCCA | TCTGATCGTT  | GGCAACCAGC |
|      | GCCATTACCG | TGCGTAACGC | GGGTGCGGGT | AGACTAGCAA  | CCGTTGGTCG |
| 1951 | ATCGCAGTGG | GAACGATGCC | CTCATTCAGC | ATTTGTCATGG | TTTGTTGAAA |
|      | TAGCGTCACC | CTTGCTACGG | GAGTAAGTCG | TAAACGTACC  | AAACAACTTT |
| 2001 | ACCGGACATG | GCACTCCAGT | CGCCTTCCCG | TTCCGCTATC  | GGCTGAATTT |
|      | TGGCCTGTAC | CGTGAGGTCA | GCGGAAGGGC | AAGGCGATAG  | CCGACTTAAA |
| 2051 | GATTGCGAGT | GAGATATTTA | TGCCAGCCAG | CCAGACGCAG  | ACGCGCCGAG |
|      | CTAACGCTCA | CTCTATAAAT | ACGGTCGGTC | GGTCTGCGTC  | TGCGCGGCTC |
| 2101 | ACAGAACTTA | ATGGGCCAGC | TAACAGCGCG | ATTTGCTGGT  | GGCCCAATGC |
|      | TGTCTTGAAT | TACCCGGTCG | ATTGTCGCGC | TAAACGACCA  | CCGGGTACG  |
| 2151 | GACCAGATGC | TCCACGCCCA | GTCGCGTACC | GTCCTCATGG  | GAGAAAATAA |
|      | CTGGTCTACG | AGGTGCGGGT | CAGCGCATGG | CAGGAGTACC  | CTCTTTTATT |
| 2201 | TACTGTTGAT | GGGTGTCTGG | TCAGAGACAT | CAAGAAATAA  | CGCCGGAACA |
|      | ATGACAATA  | CCCACAGACC | AGTCTCTGTA | GTTCTTTATT  | GCGGCCTTGT |
| 2251 | TTAGTGCAGG | CAGCTTCCAC | AGCAATAGCA | TCCTGGTCAT  | CCAGCGGATA |
|      | AATCACGTCC | GTCGAAGGTG | TCGTTATCGT | AGGACCAGTA  | GGTCGCCTAT |
|      |            |            |            | ApaLI       |            |
|      |            |            |            | ~~~~~       |            |
| 2301 | GTTAATAATC | AGCCCACTGA | CACGTTGCGC | GAGAAGATTG  | TGCACCGCCG |
|      | CAATTATTAG | TCGGGTGACT | GTGCAACGCG | CTCTTCTAAC  | ACGTGGCGGC |
| 2351 | CTTTACAGGC | TTCGACGCCG | CTTCGTTCTA | CCATCGACAC  | GACCACGCTG |
|      | GAAATGTCCG | AAGCTGCGGC | GAAGCAAGAT | GGTAGCTGTG  | CTGGTGCGAC |

|      |                           |                           |                           |                          |                          |
|------|---------------------------|---------------------------|---------------------------|--------------------------|--------------------------|
| 2401 | GCACCCAGTT<br>CGTGGGTCAA  | GATCGGCGCG<br>CTAGCCGCGC  | AGATTTAATC<br>TCTAAATTAG  | GCCGCGACAA<br>CGGCGCTGTT | TTTGCGACGG<br>AAACGCTGCC |
| 2451 | CGCGTGCAGG<br>GCGCACGTCC  | GCCAGACTGG<br>CGGTCTGACC  | AGGTGGCAAC<br>TCCACCGTTG  | GCCAATCAGC<br>CGGTTAGTCG | AACGACTGTT<br>TTGCTGACAA |
| 2501 | TGCCCCGCCAG<br>ACGGGCGGTC | TTGTTGTGCC<br>AACAAACACGG | ACGCGGTTAG<br>TGCGCCAATC  | GAATGTAATT<br>CTTACATTAA | CAGCTCCGCC<br>GTCGAGGCGG |
| 2551 | ATCGCCGCTT<br>TAGCGGCGAA  | CCACTTTTTTC<br>GGTGAAAAAG | CCGCGTTTTTC<br>GGCGCAAAAG | GCAGAAACGT<br>CGTCTTTGCA | GGCTGGCCTG<br>CCGACCGGAC |
| 2601 | G TTCACCACG<br>CAAGTGGTGC | CGGGAAACGG<br>GCCCTTTGCC  | TCTGATAAGA<br>AGACTATTCT  | GACACCGGCA<br>CTGTGGCCGT | TACTCTGCGA<br>ATGAGACGCT |
| 2651 | CATCGTATAA<br>GTAGCATATT  | CGTTACTGGT<br>GCAATGACCA  | TTCACATTCA<br>AAGTGTAAGT  | CCACCCTGAA<br>GGTGGGACTT | TTGACTCTCT<br>AACTGAGAGA |
| 2701 | TCCGGGCGCT<br>AGGCCCGCGA  | ATCATGCCAT<br>TAGTACGGTA  | ACCGCGAAAG<br>TGGCGCTTTC  | GTTTTGCGCC<br>CAAAACGCGG | ATTCGATGCT<br>TAAGCTACGA |
| 2751 | AGCCATGTGA<br>TCGGTACACT  | GCAAAAGGCC<br>CGTTTTTCCGG | AGCAAAAGGC<br>TCGTTTTTCCG | CAGGAACCGT<br>GTCCTTGGA  | AAAAAGGCCG<br>TTTTTCCGGC |
| 2801 | CGTTGCTGGC<br>GCAACGACCG  | GTTTTTCCAT<br>CAAAAAGGTA  | AGGCTCCGCC<br>TCCGAGGCGG  | CCCCTGACGA<br>GGGGACTGCT | GCATCACAAA<br>CGTAGTGTTT |
| 2851 | AATCGACGCT<br>TTAGCTGCGA  | CAAGTCAGAG<br>GTTCAGTCTC  | GTGGCGAAAC<br>CACCGCTTTG  | CCGACAGGAC<br>GGCTGTCCTG | TATAAAGATA<br>ATATTTCTAT |
| 2901 | CCAGGCGTTT<br>GGTCCGCAAA  | CCCCCTGGAA<br>GGGGGACCTT  | GCTCCCTCGT<br>CGAGGGAGCA  | GCGCTCTCCT<br>CGCGAGAGGA | GTTCCGACCC<br>CAAGGCTGGG |
| 2951 | TGCCGCTTAC<br>ACGGCGAATG  | CGGATACCTG<br>GCCTATGGAC  | TCCGCCTTTC<br>AGGCGGAAAG  | TCCCTTCGGG<br>AGGGAAGCCC | AAGCGTGGCG<br>TTCGCACCGC |
| 3001 | CTTTCTCATA<br>GAAAGAGTAT  | GCTCACGCTG<br>CGAGTGCGAC  | TAGGTATCTC<br>ATCCATAGAG  | AGTTCGGTGT<br>TCAAGCCACA | AGGTCGTTCG<br>TCCAGCAAGC |

## ApaLI

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3051	CTCCAAGCTG GAGGTTTCGAC	GGCTGTGTGC CCGACACACG	ACGAACCCCC TGCTTGGGGG	CGTTCAGCCC GCAAGTCGGG	GACCGCTGCG CTGGCGACGC
3101	CCTTATCCGG GGAATAGGCC	TAACTATCGT ATTGATAGCA	CTTGAGTCCA GAACTCAGGT	ACCCGGTAAG TGGGCCATTC	ACACGACTTA TGTGCTGAAT
3151	TCGCCACTGG AGCGGTGACC	CAGCAGCCAC GTCGTCCGGT	TGGTAACAGG ACCATGTGCC	ATTAGCAGAG TAATCGTCTC	CGAGGTATGT GCTCCATACA
3201	AGGCGGTGCT TCCGCCACGA	ACAGAGTTCT TGTCTCAAGA	TGAAGTGGTG ACTTCACCAC	GCCTAACTAC CGGATTGATG	GGCTACACTA CCGATGTGAT
3251	GAAGAACAGT CTTCTTGTCA	ATTTGGTATC TAAACCATAG	TGCGCTCTGC ACGCGAGACG	TGTAGCCAGT ACATCGGTCA	TACCTTCGGA ATGGAAGCCT

3301	AAAAGAGTTG TTTTCTCAAC	GTAGCTCTTG CATCGAGAAC	ATCCGGCAAA TAGGCCGTTT	CAAACCACCG GTTTGGTGGC	CTGGTAGCGG GACCATCGCC
3351	TGGTTTTTTT ACCAAAAAAA	GTTTGCAAGC CAAACGTTTC	AGCAGATTAC TCGTCTAATG	GCGCAGAAAA CGCGTCTTTT	AAAGGATCTC TTTCCTAGAG
3401	AAGAAGATCC TTCTTCTAGG	TTTGATCTTT AAACTAGAAA	TCTACGGGGT AGATGCCCCA	CTGACGCTCA GACTGCGAGT	GTGGAACGAA CACCTTGCTT
3451	AACTCACGTT TTGAGTGCAA	AAGGGATTTT TTCCCTAAAA	GGTCAGATCT CCAGTCTAGA	AGCACCAGGC TCGTGGTCCG	GTTTAAGGGC CAAATTCCCG
3501	ACCAATAACT TGGTTATTGA	GCCTTAAAAA CGGAATTTTT	AATTACGCCC TTAATGCGGG	CGCCCTGCCA GCGGGACGGT	CTCATCGCAG GAGTAGCGTC
3551	TACTGTTGTA ATGACAACAT	ATTCATTAAG TAAGTAATTC	CATTCTGCCG GTAAGACGGC	ACATGGAAGC TGTACCTTCG	CATCACAAAC GTAGTGTTTG
3601	GGCATGATGA CCGTACTACT	ACCTGAATCG TGGACTTAGC	CCAGCGGCAT GGTCGCCGTA	CAGCACCTTG GTCGTGGAAC	TCGCCCTTGCG AGCGGAACGC
3651	TATAATATTT ATATTATAAA	GCCCATAGTG CGGGTATCAC	AAAACGGGGG TTTTTGCCCC	CGAAGAAGTT GCTTCTTCAA	GTCCATATTG CAGGTATAAC
3701	GCTACGTTTA CGATGCAAAT	AATCAAAACT TTAGTTTTGA	GGTGAAACTC CCACTTTGAG	ACCCAGGGAT TGGGTCCCTA	TGGCTGAGAC ACCGACTCTG
3751	GAAAAACATA CTTTTTGTAT	TTCTCAATAA AAGAGTTATT	ACCCTTTAGG TGGGAAATCC	GAAATAGGCC CTTTATCCGG	AGGTTTTTAC TCCAAAAGTG
3801	CGTAACACGC GCATTGTGCG	CACATCTTGC GTGTAGAACG	GAATATATGT CTTATATACA	GTAGAAACTG CATCTTTGAC	CCGGAAATCG GGCCTTTAGC
3851	TCGTGGTATT AGCACCATAA	CACTCCAGAG GTGAGGTCTC	CGATGAAAAC GCTACTTTTT	GTTTCAGTTT CAAAGTCAA	GCTCATGGAA CGAGTACCTT
3901	AACGGTGTA TTGCCACATT	CAAGGGTGAA GTTCCCACTT	CACTATCCCA GTGATAGGGT	TATCACCAGC ATAGTGGTCG	TCACCGTCTT AGTGGCAGAA
3951	TCATTGCCAT AGTAACGGTA	ACGGAACCTC TGCCTTGAGG	GGGTGAGCAT CCCACTCGTA	TCATCAGGCG AGTAGTCCGC	GGCAAGAATG CCGTTCTTAC
4001	TGAATAAAGG ACTTATTTCC	CCGGATAAAA GGCCTATTTT	CTTGTGCTTA GAACACGAAT	TTTTTCTTTA AAAAAGAAAT	CGGTCTTTAA GCCAGAAATT
4051	AAAGGCCGTA TTTCCGGCAT	ATATCCAGCT TATAGGTCGA	GAACGGTCTG CTTGCCAGAC	GTTATAGGTA CAATATCCAT	CATTGAGCAA GTAACCTGTT
4101	CTGACTGAAA GACTGACTTT	TGCCTCAAAA ACGGAGTTTT	TGTTCTTTAC ACAAGAAATG	GATGCCATTG CTACGGTAAC	GGATATATCA CCTATATAGT
4151	ACGGTGGTAT TGCCACCATA	ATCCAGTGAT TAGGTCACCT	TTTTTTCTCC AAAAAAGAGG	ATTTTAGCTT TAAAATCGAA	CCTTAGCTCC GGAATCGAGG
4201	TGAAAATCTC ACTTTTAGAG	GATAACTCAA CTATTGAGTT	AAAATACGCC TTTTATGCGG	CGGTAGTGAT GCCATCACTA	CTTATTTTCAT GAATAAAGTA

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4251 TATGGTGAAA GTTGGAACCT CACCCGACGT CTAATGTGAG TTAGCTCACT  
ATACCACTTT CAACCTTGGA GTGGGCTGCA GATTACACTC AATCGAGTGA

4301 CATTAGGCAC CCCAGGCTTT ACACTTTATG CTTCCGGCTC GTATGTTGTG  
GTAATCCGTG GGGTCCGAAA TGTGAAATAC GAAGGCCGAG CATAACAACAC

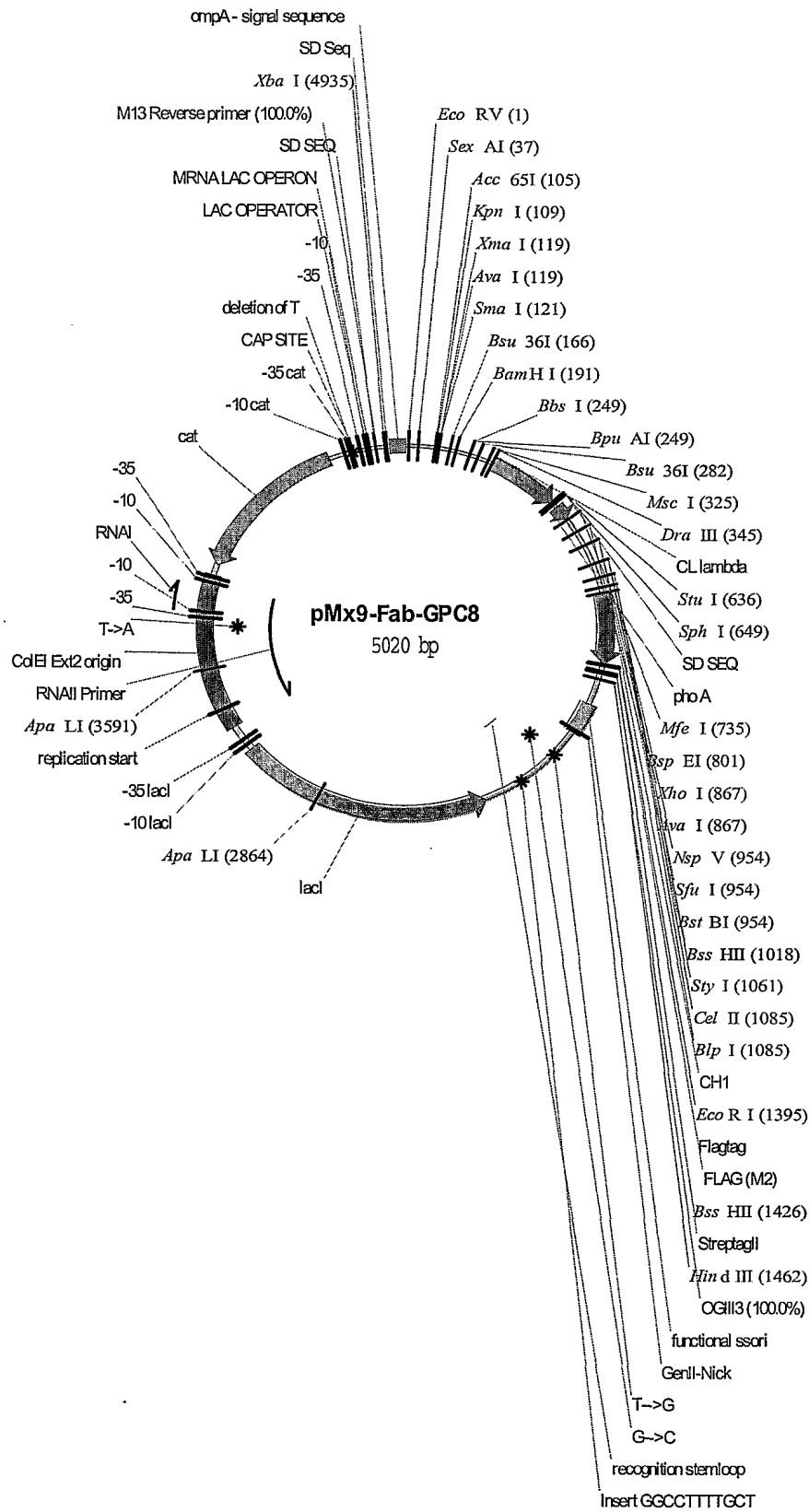
M13 Reverse primer 100.0%

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4351 TGGAATTGTG AGCGGATAAC AATTTACAC AGGAAACAGC TATGACCATG  
ACCTTAACAC TCGCCTATTG TTAAAGTGTG TCCTTTGTCTG ATACTGGTAC

4401 ATTACGAATT  
TAATGCTTAA

Figure 13



# Figure 13 (cont)

	EcoRV				SexAI
	~~~~~				~~~~~
1	ATCGTGCTGA	CCCAGCCGCC	TTCAGTGAGT	GGCGCACCAG	GTCAGCGTGT
	TAGCACGACT	GGGTCGGCGG	AAGTCACTCA	CCGCGTGGTC	CAGTCGCACA
51	GACCATCTCG	TGTAGCGGCA	GCAGCAGCAA	CATTGGCAGC	AACTATGTGA
	CTGGTAGAGC	ACATCGCCGT	CGTCGTCGTT	GTAACCGTCG	TTGATACT
		XmaI			
		~~~~~			
	KpnI	SmaI			
	~~~~~	~~~~~			
	Acc65I	AvaI			
	~~~~~	~~~~~			
101	GCTGGTACCA	GCAGTTGCCC	GGGACGGCGC	CGAAACTGCT	GATTTATGAT
	CGACCATGGT	CGTCAACGGG	CCCTGCCGCG	GCTTTGACGA	CTAAATACTA
		Bsu36I		BamHI	
		~~~~~		~~~~~	
151	AACAACCAGC	GTCCCTCAGG	CGTGCCGGAT	CGTTTTAGCG	GATCCAAAAG
	TTGTTGGTCG	CAGGGAGTCC	GCACGGCCTA	GCAAAATCGC	CTAGGTTTTTC
				BpuAI	
				~~~~~	
				BbsI	
				~~~~~	
201	CGGCACCAGC	GCGAGCCTTG	CGATTACGGG	CCTGCAAAGC	GAAGACGAAG
	GCCGTGGTCG	CGCTCGGAAC	GCTAATGCCC	GGACGTTTTCG	CTTCTGCTTC
		Bsu36I			
		~~~~~			
251	CGGATTATTA	TTGCCAGAGC	TATGACATGC	CTCAGGCTGT	GTTTGGCGGC
	GCCTAATAAT	AACGGTCTCG	ATACTGTACG	GAGTCCGACA	CAAACCGCCG
		MscI		DraIII	
		~~~~~		~~~~~	
301	GGCACGAAGT	TTAACCGTTC	TTGGCCAGCC	GAAAGCCGCA	CCGAGTGTGA
	CCGTGCTTCA	AATTGGCAAG	AACCGGTCGG	CTTTCGGCGT	GGCTCACACT
351	CGCTGTTTCC	GCCGAGCAGC	GAAGAATTGC	AGGCGAACAA	AGCGACCCTG
	GCGACAAAGG	CGGCTCGTCG	CTTCTTAACG	TCCGCTTGTT	TCGCTGGGAC
401	GTGTGCCTGA	TTAGCGACTT	TTATCCGGGA	GCCGTGACAG	TGGCCTGGAA
	CACACGGACT	AATCGCTGAA	AATAGGCCCT	CGGCACTGTC	ACCGGACCTT
451	GGCAGATAGC	AGCCCCGTCA	AGGCGGGAGT	GGAGACCACC	ACACCCTCCA
	CCGTCTATCG	TCGGGGCAGT	TCCGCCCTCA	CCTCTGGTGG	TGTGGGAGGT
501	AACAAAGCAA	CAACAAGTAC	GCGGCCAGCA	GCTATCTGAG	CCTGACGCCT
	TTGTTTTCGTT	GTTGTTCATG	CGCCGGTCGT	CGATAGACTC	GGACTGCGGA
551	GAGCAGTGGA	AGTCCCACAG	AAGCTACAGC	TGCCAGGTCA	CGCATGAGGG
	CTCGTCACCT	TCAGGGTGTC	TTCGATGTCG	ACGGTCCAGT	GCGTACTCCC
			StuI		SphI

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        ~~~~~~
601  GAGCACCGTG  GAAAAAACCG  TTGCGCCGAC  TGAGGCCTGA  TAAGCATGCG
      CTCGTGGCAC  CTTTTTTGGC  AACGCGGCTG  ACTCCGGACT  ATTCGTACGC

651  TAGGAGAAAA  TAAAATGAAA  CAAAGCACTA  TTGCACTGGC  ACTCTTACCG
      ATCCTCTTTT  ATTTTACTTT  GTTTCGTGAT  AACGTGACCG  TGAGAATGGC

                                MfeI
                                ~~~~~~
701  TTGCTCTTCA  CCCCTGTTAC  CAAAGCCCAG  GTGCAATTGA  AAGAAAGCGG
      AACGAGAAAGT  GGGGACAATG  GTTTCGGGTC  CACGTTAACT  TTCTTTCGCC

                                BspEI
                                ~~~~~~
751  CCCGGCCCTG  GTGAAACCGA  CCCAAACCCT  GACCCTGACC  TGTACCTTTT
      GGGCCGGGAC  CACTTTGGCT  GGGTTTGGGA  CTGGGACTGG  ACATGGAAAA

      BspEI
      ~~~~~~
801  CCGGATTTAG  CCTGTCCACG  TCTGGCGTTG  GCGTGGGCTG  GATTCGCCAG
      GGCCTAAATC  GGACAGGTGC  AGACCGCAAC  CGCACCCGAC  CTAAGCGGTC

                                XhoI
                                ~~~~~~
                                AvaI
                                ~~~~~~
851  CCGCCTGGGA  AAGCCCTCGA  GTGGCTGGCT  CTGATTGATT  GGGATGATGA
      GGCGGACCCT  TTCGGGAGCT  CACCGACCGA  GACTAACTAA  CCCTACTACT

901  TAAGTATTAT  AGCACCAGCC  TGAAAACGCG  TCTGACCATT  AGCAAAGATA
      ATTCATAATA  TCGTGGTCCG  ACTTTTGCGC  AGACTGGTAA  TCGTTTCTAT

      BstBI
      ~~~~~~
      SfuI
      ~~~~~~
      NspV
      ~~~~~~
951  CTTCGAAAAA  TCAGGTGGTG  CTGACTATGA  CCAACATGGA  CCCGGTGGAT
      GAAGCTTTTT  AGTCCACCAC  GACTGATACT  GGTGTACCT  GGGCCACCTA

                                BssHII
                                ~~~~~~
1001  ACGGCCACCT  ATTATTGCGC  GCGTTCTCCT  CGTTATCGTG  GTGCTTTTGA
      TGCCGGTGGA  TAATAACGCG  CGCAAGAGGA  GCAATAGCAC  CACGAAAAC

                                BspI
                                ~~~~~~
                                CelII
                                ~~~~~~
                                StyI
                                ~~~~~~
1051  TTATTGGGGC  CAAGGCACCC  TGGTGACGGT  TAGCTCAGCG  TCGACCAAAG
      AATAACCCCG  GTTCCGTGGG  ACCACTGCCA  ATCGAGTCGC  AGCTGGTTTC

1101  GTCCAAGCGT  GTTTCGGCTG  GCTCCGAGCA  GCAAAAGCAC  CAGCGGCGGC
      CAGGTTCGCA  CAAAGGCGAC  CGAGGCTCGT  CGTTTTCGTG  GTCGCCGCCG

1151  ACGGCTGCCC  TGGGCTGCCT  GGTAAAGAT  TATTTCCCGG  AACCAGTCAC

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	TGCCGACGGG	ACCCGACGGA	CCAATTTCTA	ATAAAGGGCC	TTGGTCAGTG
1201	CGTGAGCTGG GCACTCGACC	AACAGCGGGG TTGTCGCCCC	CGCTGACCAG GCGACTGGTC	CGGCGTG CAT GCCGCACGTA	ACCTTTCCGG TGGAAAGGCC
1251	CGGTGCTGCA GCCACGACGT	AAGCAGCGGC TTCGTCGCCG	CTGTATAGCC GACATATCGG	TGAGCAGCGT ACTCGTCGCA	TGTGACCGTG ACACTGGCAC
1301	CCGAGCAGCA GGCTCGTCGT	GCTTAGGCAC CGAATCCGTG	TCAGACCTAT AGTCTGGATA	ATTTGCAACG TAAACGTTGC	TGAACCATAA ACTTGGTATT
				EcoRI ~~~~~	
1351	ACCGAGCAAC TGGCTCGTTG	ACCAAAGTGG TGGTTTCACC	ATAAAAAAGT TATTTTTTCA	GGAACCGAAA CCTTGGCTTT	AGCGAATTCG TCGCTTAAGC
			BssHII ~~~~~		
1401	ACTATAAAGA TGATATTTCT	TGACGATGAC ACTGCTACTG	AAAGGCGCGC TTTCCGCGCG	CGTGGAGCCA GCACCTCGGT	CCCGCAGTTT GGGCGTCAAA
		HindIII ~~~~~			
1451	GAAAAATGAT CTTTTTACTA	AAGCTTGACC TTCGAACTGG	TGTGAAGTGA ACACTTCACT	AAAATGGCGC TTTTACCGCG	AGATTGTGCG TCTAACACGC
			OGIII3 100.0%		
			=====		
1501	ACATTTTTTTT TGTAACAAAA	TGTCTGCCGT ACAGACGGCA	TTAATTAAAG AATTAATTTT	GGGGGGGGGG CCCCCCCCC	GCCGGCCTGG CGGCCGGACC
1551	GGGGGGGTGT CCCCCCCACA	ACATGAAATT TGTACTTTAA	GTAAACGTTA CATTTGCAAT	ATATTTTGTT TATAAAACAA	AAAATTCGCG TTTTAAGCGC
1601	TTAAATTTTTT AATTTAAAAA	GTTAAATCAG CAATTTAGTC	CTCATTTTTT GAGTAAAAAA	AACCAATAGG TTGGTTATCC	CCGAAATCGG GGCTTTAGCC
1651	CAAAATCCCT GTTTTAGGGA	TATAAATCAA ATATTTAGTT	AAGAATAGAC TTCTTATCTG	CGAGATAGGG GCTCTATCCC	TTGAGTGTTG AACTCACAA
1701	TTCCAGTTTG AAGGTCAAAC	GAACAAGAGT CTTGTTCTCA	CCACTATTAA GGTGATAATT	AGAACGTGGA TCTTGACACT	CTCCAACGTC GAGGTTGCAG
1751	AAAGGGCGAA TTTCCCGCTT	AAACCGTCTA TTTGGCAGAT	TCAGGGCGAT AGTCCCGCTA	GGCCCACTAC CCGGGTGATG	GAGAACCATC CTCTTGGTAG
1801	ACCCTAATCA TGGGATTAGT	AGTTTTTTTGG TCAAAAAACC	GGTCGAGGTG CCAGCTCCAC	CCGTAAAGCA GGCATTTTCG	CTAAATCGGA GATTTAGCCT
1851	ACCCTAAAGG TGGGATTTCC	GAGCCCCCGA CTCGGGGGCT	TTTAGAGCTT AAATCTCGAA	GACGGGGAAA CTGCCCTTTT	GCCGGCGAAC CGGCCGCTTG
1901	GTGGCGAGAA CACCGCTCTT	AGGAAGGGAA TCCTTCCCTT	GAAAGCGAAA CTTTCGCTTT	GGAGCGGGCG CCTCGCCCCG	CTAGGGCGCT GATCCCGCGA
1951	GGCAAGTGTA CCGTTCACAT	GCGGTCACGC CGCCAGTGCG	TGCGCGTAAC ACGCGCATTG	CACCACACCC GTGGTGTGGG	GCCGCGCTTA CGGCGCGAAT

2001	ATGCGCCGCT TACGCGGCGA	ACAGGGCGCG TGTCCC GCGC	TGCTAGACTA ACGATCTGAT	GTGTTTAAAC CACAAATTTG	CGGACCGGGG GCCTGGCCCC
2051	GGGGGCTTAA CCCCCGAATT	GTGGGCTGCA CACCCGACGT	AAACAAAACG TTTGT TTTGC	GCCTCCTGTC CGGAGGACAG	AGGAAGCCGC TCCTTCGGCG
2101	TTTTATCGGG AAAATAGCCC	TAGCCTCACT ATCGGAGTGA	GCCCGCTTTC CGGGCGAAAG	CAGTCGGGAA GTCAGCCCTT	ACCTGTCGTG TGGACAGCAC
2151	CCAGCTGCAT GGTCGACGTA	CAGTGAATCG GTCAC TTAGC	GCCAACGCGC CGGTTGCGCG	GGGGAGAGGC CCCCTCTCCG	GGTTTGC GTA CCAAACGCAT
2201	TTGGGAGCCA AACCCTCGGT	GGGTGGTTTT CCCACCAAAA	TCTTTTCACC AGAAAAGTGG	AGTGAGACGG TCACTCTGCC	GCAACAGCTG CGTTGTCGAC
2251	ATTGCCCTTC TAACGGGAAG	ACCGCCTGGC TGGCGGACCG	CCTGAGAGAG GGACTCTCTC	TTGCAGCAAG AACGTCGTTC	CGGTCCACGC GCCAGGTGCG
2301	TGGTTTGCCC ACCAAACGGG	CAGCAGGCGA GTCGTCCGCT	AAATCCTGTT TTTAGGACAA	TGATGGTGGT ACTACCACCA	CAGCGGCGGG GTCGCCGCC
2351	ATATAACATG TATATTGTAC	AGCTGTCCTC TCGACAGGAG	GGTATCGTCG CCATAGCAGC	TATCCC ACTA ATAGGGTGAT	CCGAGATGTC GGCTCTACAG
2401	CGCACCAACG GCGTG GTTG	CGCAGCCCCG GCGTCGGGCC	ACTCGGTAAT TGAGCCATTA	GGCACGCATT CCGTGCGTAA	GCGCCCAGCG CGCGGGTCGC
2451	CCATCTGATC GGTAGACTAG	GTTGGCAACC CAACCGTTGG	AGCATCGCAG TCGTAGCGTC	TGGGAACGAT ACCCTTGCTA	GCCCTCATTC CGGGAGTAAG
2501	AGCATTTGCA TCGTAAACGT	TGGTTTGTTG ACCAAACAAC	AAAACCGGAC TTT TGGCCTG	ATGGCACTCC TACCGTGAGG	AGTCGCCTTC TCAGCGGAAG
2551	CCGTTCCGCT GGCAAGGCGA	ATCGGCTGAA TAGCCGACTT	TTTGATTGCG AAACTAACGC	AGTGAGATAT TCACTCTATA	TTATGCCAGC AATACGGTCG
2601	CAGCCAGACG GTCGGTCTGC	CAGACGCGCC GTCTGCGCGG	GAGACAGAAC CTCTGTCTTG	TTAATGGGCC AATTACCCGG	AGCTAACAGC TCGATTGTGC
2651	GCGATTTGCT CGCTAAACGA	GGTGGCCCAA CCACCGGGTT	TGCGACCAGA ACGCTGGTCT	TGCTCCACGC ACGAGGTGCG	CCAGTCGCGT GGTCAGCGCA
2701	ACCGTCCTCA TGGCAGGAGT	TGGGAGAAAA ACCCTCTTTT	TAATACTGTT ATTATGACAA	GATGGGTGTC CTACCCACAG	TGGTCAGAGA ACCAGTCTCT
2751	CATCAAGAAA GTAGTTCTTT	TAACGCCGGA ATTGCGGCCT	ACATTAGTGC TGTAATCACG	AGGCAGCTTC TCCGTCGAAG	CACAGCAATA GTGTCGTTAT
2801	GCATCCTGGT CGTAGGACCA	CATCCAGCGG GTAGGTCGCC	ATAGTTAATA TATCAATTAT	ATCAGCCCAC TAGTCGGGTG	TGACACGTTG ACTGTGCAAC
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2851	CGCGAGAAGA GCGCTCTTCT	TTGTGCACCG AACACGTGGC	CCGCTTTTACA GGCGAAATGT	GGCTTCGACG CCGAAGCTGC	CCGCTTCGTT GGCGAAGCAA

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2901	CTACCATCGA GATGGTAGCT	CACGACCACG GTGCTGGTGC	CTGGCACCCA GACCGTGGGT	GTTGATCGGC CAACTAGCCG	GCGAGATTTA CGCTCTAAAT
2951	ATCGCCGCGA TAGCGGCGCT	CAATTTGCGA GTTAAACGCT	CGGCGCGTGC GCCGCGCACG	AGGGCCAGAC TCCCAGTCTG	TGGAGGTGGC ACCTCCACCG
3001	AACGCCAATC TTGCGGTTAG	AGCAACGACT TCGTTGCTGA	GTTTGCCCGC CAAACGGGCG	CAGTTGTTGT GTCAACAACA	GCCACGCGGT CGGTGCGCCA
3051	TAGGAATGTA ATCCTTACAT	ATTCAGCTCC TAAGTCGAGG	GCCATCGCCG CGGTAGCGGC	CTTCCACTTT GAAGGTGAAA	TTCCCGCGTT AAGGGCGCAA
3101	TTTCGAGAAA AAGCGTCTTT	CGTGGCTGGC GCACCGACCG	CTGGTTCACC GACCAAGTGG	ACGCGGGA TGCGCCCTTT	CGGTCTGATA GCCAGACTAT
3151	AGAGACACCG TCTCTGTGGC	GCATACTCTG CGTATGAGAC	CGACATCGTA GCTGTAGCAT	TAACGTTACT ATTGCAATGA	GGTTTCACAT CCAAAGTGTA
3201	TCACCACCCT AGTGGTGGGA	GAATTGACTC CTTAACTGAG	TCTTCCGGGC AGAAGGCCCG	GCTATCATGC CGATAGTACG	CATACCGCGA GTATGGCGCT
3251	AAGGTTTTGC TTCCAAAACG	GCCATTCGAT CGGTAAGCTA	GCTAGCCATG CGATCGGTAC	TGAGCAAAAG ACTCGTTTTT	GCCAGCAAAA CGGTCTTTTT
3301	GGCCAGGAAC CCGTCCTTG	CGTAAAAAGG GCATTTTTCC	CCGCGTTGCT GGCGCAACGA	GGCGTTTTTC CCGCAAAAAG	CATAGGCTCC GTATCCGAGG
3351	GCCCCCTGA CGGGGGGACT	CGAGCATCAC GCTCGTAGTG	AAAAATCGAC TTTTTTAGCTG	GCTCAAGTCA CGAGTTCAGT	GAGGTGGCGA CTCCACCGCT
3401	AACCCGACAG TTGGGCTGTC	GACTATAAAG CTGATATTTT	ATACCAGGCG TATGGTCCGC	TTTCCCCCTG AAAGGGGGAC	GAAGCTCCCT CTTCGAGGGA
3451	CGTGCGCTCT GCACGCGAGA	CCTGTTCCGA GGACAAGGCT	CCCTGCCGCT GGGACGGCGA	TACCGGATAC ATGGCCTATG	CTGTCCGCCT GACAGGCGGA
3501	TTCTCCCTTC AAGAGGGAAG	GGGAAGCGTG CCCTTCGCAC	GCGCTTTCTC CGCGAAAGAG	ATAGCTCACG TATCGAGTGC	CTGTAGGTAT GACATCCATA
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3551	CTCAGTTCGG GAGTCAAGCC	TGTAGGTCGT ACATCCAGCA	TCGCTCCAAG AGCGAGGTTC	CTGGGCTGTG GACCCGACAC	TGCACGAACC ACGTGCTTGG
3601	CCCCGTTTAC GGGGCAAGTC	CCCGACCGCT GGGCTGGCGA	GCGCCTTATC CGCGGAATAG	CGGTAACTAT GCCATTGATA	CGTCTTGAGT GCAGAACTCA
3651	CCAACCCGGT GGTTGGGCCA	AAGACACGAC TTCTGTGCTG	TTATCGCCAC AATAGCGGTG	TGGCAGCAGC ACCGTCGTCTG	CACTGGTAAC GTGACCATTG
3701	AGGATTAGCA TCCTAATCGT	GAGCGAGGTA CTCGCTCCAT	TGTAGGCGGT ACATCCGCCA	GCTACAGAGT CGATGTCTCA	TCTTGAAGTG AGAACTTCAC
3751	GTGGCCTAAC CACCGGATTG	TACGGCTACA ATGCCGATGT	CTAGAAGAAC GATCTTCTTG	AGTATTTGGT TCATAAACCA	ATCTGCGCTC TAGACGCGAG
3801	TGCTGTAGCC	AGTTACCTTC	GGAAAAAGAG	TTGGTAGCTC	TTGATCCGGC

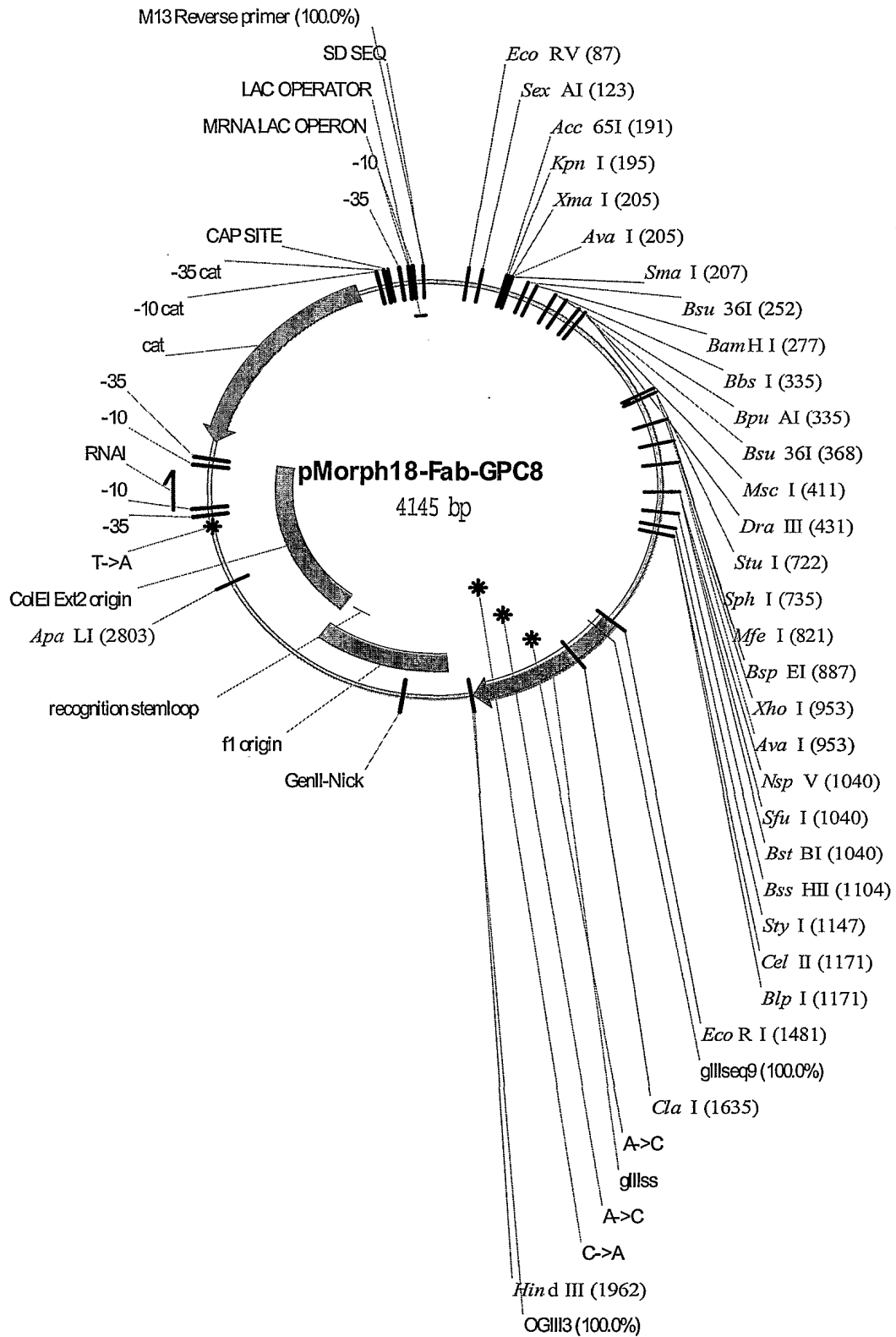
	ACGACATCGG	TCAATGGAAG	CCTTTTTTCTC	AACCATCGAG	AACTAGGCCG
3851	AAACAAACCA TTTGTTTGGT	CCGCTGGTAG GGCGACCATC	CGGTGGTTTT GCCACCAAAA	TTTGTTTGCA AAACAAACGT	AGCAGCAGAT TCGTCGTCTA
3901	TACGCGCAGA ATGCGCGTCT	AAAAAAGGAT TTTTTTCCTA	CTCAAGAAGA GAGTTCCTCT	TCCTTTGATC AGGAAACTAG	TTTTCTACGG AAAAGATGCC
3951	GGTCTGACGC CCAGACTGCG	TCAGTGGAAC AGTCACCTTG	GAAAACTCAC CTTTTGAGTG	GTTAAGGGAT CAATTCCCTA	TTTGGTCAGA AAACCAGTCT
4001	TCTAGCACCA AGATCGTGGT	GGCGTTTAAG CCGCAAATTC	GGCACCAATA CCGTGGTTAT	ACTGCCTTAA TGACGGAATT	AAAAATTACG TTTTTAATGC
4051	CCCCGCCCTG GGGGCGGGAC	CCACTCATCG GGTGAGTAGC	CAGTACTGTT GTCATGACAA	GTAATTCATT CATTAAGTAA	AAGCATTCTG TTCGTAAGAC
4101	CCGACATGGA GGCTGTACCT	AGCCATCACA TCGGTAGTGT	AACGGCATGA TTGCCGTACT	TGAACCTGAA ACTTGGAATT	TCGCCAGCGG AGCGGTCGCC
4151	CATCAGCACC GTAGTCGTGG	TTGTGCGCTT AACAGCGGAA	GCGTATAATA CGCATATTAT	TTTGCCCATTA AAACGGGTAT	GTGAAAACGG CACTTTTGCC
4201	GGGCGAAGAA CCCCTTCTT	GTTGTCCATA CAACAGGTAT	TTGGCTACGT AACCGATGCA	TTAAATCAAA AATTTAGTTT	ACTGGTGAAA TGACCACTTT
4251	CTCACCAGG GAGTGGGTCC	GATTGGCTGA CTAACCGACT	GACGAAAAAC CTGCTTTTTG	ATATTCTCAA TATAAGAGTT	TAAACCCTTT ATTTGGGAAA
4301	AGGGAAATAG TCCCTTTATC	GCCAGGTTTT CGGTCCAAAA	CACCGTAACA GTGGCATTGT	CGCCACATCT GCGGTGTAGA	TGCGAATATA ACGCTTATAT
4351	TGTGTAGAAA ACACATCTTT	CTGCCGGAAA GACGGCCTTT	TCGTGCTGGT AGCAGCACCA	ATTCACCTCA TAAGTGAGGT	GAGCGATGAA CTCGCTACTT
4401	AACGTTTCAG TTGCAAAGTC	TTTGCTCATG AAACGAGTAC	GAAAACGGTG CTTTTGCCAC	TAACAAGGGT ATTGTTCCCA	GAACACTATC CTTGTGATAG
4451	CCATATCACC GGTATAGTGG	AGCTCACCGT TCGAGTGGCA	CTTTCATTGC GAAAGTAACG	CATACGGAAC GTATGCCTTG	TCCGGGTGAG AGGCCCACTC
4501	CATTTCATCAG GTAAGTAGTC	GCGGGCAAGA CGCCCGTTCT	ATGTGAATAA TACACTTATT	AGGCCGGATA TCCGGCCTAT	AAACTTGTGC TTTGAACACG
4551	TTATTTTTTCT AATAAAAAGA	TTACGGTCTT AATGCCAGAA	TAAAAAGGCC ATTTTTCCGG	GTAATATCCA CATTATAGGT	GCTGAACGGT CGACTTGCCA
4601	CTGGTTATAG GACCAATATC	GTACATTGAG CATGTAACCT	CAACTGACTG GTTGACTGAC	AAATGCCTCA TTTACGGAGT	AAATGTTCTT TTTACAAGAA
4651	TACGATGCCA ATGCTACGGT	TTGGGATATA AACCCTATAT	TCAACGGTGG AGTTGCCACC	TATATCCAGT ATATAGGTCA	GATTTTTTTT CTAAAAAAG
4701	TCCATTTTATG AGGTAAAATC	CTTCCTTAGC GAAGGAATCG	TCCTGAAAAT AGGACTTTTA	CTCGATAACT GAGCTATTGA	CAAAAAATAC GTTTTTTATG
4751	GCCCGGTAGT	GATCTTATTT	CATTATGGTG	AAAGTTGGAA	CCTCACCCGA

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CGGGCCATCA CTAGAATAAA GTAATACCAC TTTCAACCTT GGAGTGGGCT
4801 CGTCTAATGT GAGTTAGCTC ACTCATTAGG CACCCCAGGC TTTACACTTT
GCAGATTACA CTCAATCGAG TGAGTAATCC GTGGGGTCCG AAATGTGAAA
4851 ATGCTTCCGG CTCGTATGTT GTGTGGAATT GTGAGCGGAT AACAAATTTCA
TACGAAGGCC GAGCATACAA CACACCTTAA CACTCGCCTA TTGTTAAAGT

M13 Reverse primer 100.0% XbaI
===== ~~~~~~
4901 CACAGGAAAC AGCTATGACC ATGATTACGA ATTTCTAGAT AACGAGGGCA
GTGTCCTTTG TCGATACTGG TACTAATGCT TAAAGATCTA TTGCTCCCGT
4951 AAAAATGAAA AAGACAGCTA TCGCGATTGC AGTGGCACTG GCTGGTTTCG
TTTTTACTTT TTCTGTCGAT AGCGCTAACG TCACCGTGAC CGACCAAAGC

EcoRV
~~~
5001 CTACCGTAGC GCAGGCCGAT
GATGGCATCG CGTCCGGCTA
```

**Figure 14**

**Figure 14 (cont)**

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1   TCAGATAACG AGGGCAAAAA ATGAAAAAGA CAGCTATCGC GATTGCAGTG
    AGTCTATTGC TCCCGTTTTT TACTTTTTCT GTCGATAGCG CTAACGTCAC

                                EcoRV
                                ~~~~~~
51   GCACTGGCTG GTTTCGCTAC CGTAGCGCAG GCCGATATCG TGCTGACCCA
    CGTGACCGAC CAAAGCGATG GCATCGCGTC CGGCTATAGC ACGACTGGGT

                                SexAI
                                ~~~~~~
101  GCCGCCTTCA GTGAGTGGCG CACCAGGTCA GCGTGTGACC ATCTCGTGTA
    CGGCGGAAGT CACTCACCGC GTGGTCCAGT CGCACACTGG TAGAGCACAT

                                KpnI
                                ~~~~~~
                                Acc65I
                                ~~~~~~
151  GCGGCAGCAG CAGCAACATT GGCAGCAACT ATGTGAGCTG GTACCAGCAG
    CGCCGTCGTC GTCGTTGTAA CCGTCGTTGA TACACTCGAC CATGGTCGTC

    XmaI
    ~~~~~~
    SmaI
    ~~~~~~
    AvaI
    ~~~~~~
                                Bsu36I
                                ~~~~~~
201  TTGCCCCGGA CGGCGCCGAA ACTGCTGATT TATGATAACA ACCAGCGTCC
    AACGGGCCCT GCCGCGGCTT TGACGACTAA ATACTATTGT TGGTCGCAGG

    Bsu36I
    ~~~~~~
                                BamHI
                                ~~~~~~
251  CTCAGGCGTG CCGGATCGTT TTAGCGGATC CAAAAGCGGC ACCAGCGCGA
    GAGTCCGCAC GGCCTAGCAA AATCGCCTAG GTTTTCGCCG TGGTCGCGCT

                                BpuAI
                                ~~~~~~
                                BbsI
                                ~~~~~~
301  GCCTTGCGAT TACGGGCCTG CAAAGCGAAG ACGAAGCGGA TTATTATTGC
    CGGAACGCTA ATGCCCGGAC GTTTCGCTTC TGCTTCGCCT AATAATAACG

                                Bsu36I
                                ~~~~~~
351  CAGAGCTATG ACATGCCTCA GGCTGTGTTT GGCGGCGGCA CGAAGTTTAA
    GTCTCGATAC TGTACGGAGT CCGACACAAA CCGCCGCCGT GCTTCAAATT

    MscI
    ~~~~~~
                                DraIII
                                ~~~~~~
401  CCGTTCCTTG CCAGCCGAAA GCCGCACCGA GTGTGACGCT GTTTCGCCCG
    GGCAAGAACC GGTCTGGCTTT CGGCGTGGCT CACACTGCGA CAAAGGCGGC

451  AGCAGCGAAG AATTGCAGGC GAACAAAGCG ACCCTGGTGT GCCTGATTAG
    TCGTCGCTTC TTAACGTCCG CTTGTTTCGC TGGGACCACA CGGACTAATC

501  CGACTTTTAT CCGGGAGCCG TGACAGTGGC CTGGAAGGCA GATAGCAGCC

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      GCTGAAAATA GGCCTCGGC ACTGTCACCG GACCTTCCGT CTATCGTCGG
551  CCGTCAAGGC GGGAGTGGAG ACCACCACAC CCTCCAAACA AAGCAACAAC
      GGCAGTTCCG CCCTCACCTC TGGTGGTGTG GGAGGTTTGT TTCGTTGTTG
601  AAGTACGCGG CCAGCAGCTA TCTGAGCCTG ACGCCTGAGC AGTGGAAAGTC
      TTCATGCGCC GGTCTGTCGAT AGACTCGGAC TCGCGACTCG TCACCTTCAG
651  CCACAGAAGC TACAGCTGCC AGGTCACGCA TGAGGGGAGC ACCGTGGAAA
      GGTGTCTTCG ATGTCGACGG TCCAGTGCGT ACTCCCCTCG TGGCACCTTT

      StuI              SphI
      ~~~~~~          ~~~~~~
701  AAACCGTTGC GCCGACTGAG GCCTGATAAG CATGCGTAGG AGAAAATAAA
      TTTGGCAACG CGGCTGACTC CGGACTATTC GTACGCATCC TCTTTTATTT
751  ATGAAACAAA GCACTATTGC ACTGGCACTC TTACCGTTGC TCTTCACCCC
      TACTTTGTTT CGTGATAACG TGACCGTGAG AATGGCAACG AGAAGTGGGG

      MfeI
      ~~~~~~
801  TGTTACCAAA GCCCAGGTGC AATTGAAAGA AAGCGGCCCG GCCCTGGTGA
      ACAATGGTTT CGGGTCCACG TTAACCTTCT TTCGCCGGGC CGGGACCACT

      BspEI
      ~~~~~~
851  AACCGACCCA AACCTGACC CTGACCTGTA CCTTTTCCGG ATTTAGCCTG
      TTGGCTGGGT TTGGGACTGG GACTGGACAT GGAAGAAGGCC TAAATCGGAC
901  TCCACGTCTG GCGTTGGCGT GGGCTGGATT CGCCAGCCGC CTGGGAAAGC
      AGGTGCAGAC CGCAACCGCA CCCGACCTAA GCGGTCGGCG GACCCTTTCTG

      XhoI
      ~~~~~~
      AvaI
      ~~~~~~
951  CCTCGAGTGG CTGGCTCTGA TTGATTGGGA TGATGATAAG TATTATAGCA
      GGAGCTCACC GACCGAGACT AACTAACCCT ACTACTATTC ATAATATCGT

      BstBI
      ~~~~~~
      SfuI
      ~~~~~~
      NspV
      ~~~~~~
1001 CCAGCCTGAA AACGCGTCTG ACCATTAGCA AAGATACTTC GAAAAATCAG
      GGTCGGACTT TTGCGCAGAC TGGTAATCGT TTCTATGAAG CTTTTTAGTC
1051 GTGGTGCTGA CTATGACCAA CATGGACCCG GTGGATACGG CCACCTATTA
      CACCACGACT GATACTGGTT GTACCTGGGC CACCTATGCC GGTGGATAAT

      BssHII              StyI
      ~~~~~~          ~~~~~~
1101 TTGCGCGCGT TCTCCTCGTT ATCGTGGTGC TTTTGATTAT TGGGGCCAAG
      AACGCGCGCA AGAGGAGCAA TAGCACCACG AAAACTAATA ACCCCGGTTC

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BlpI



42/49

	StyI		~~~~~		
	~		~~~~~		
			~~~~~		
1151	GCACCCTGGT CGTGGGACCA	GACGGTTAGC CTGCCAATCG	TCAGCGTCGA AGTCGCAGCT	CCAAAGGTCC GGTTTCCAGG	AAGCGTGTTT TTCGCACAAA
1201	CCGCTGGCTC GGCGACCGAG	CGAGCAGCAA GCTCGTCGTT	AAGCACCAGC TTCGTGGTCG	GGCGGCACGG CCGCCGTGCC	CTGCCCTGGG GACGGGACCC
1251	CTGCCTGGTT GACGGACCAA	AAAGATTATT TTTCTAATAA	TCCCGGAACC AGGGCCTTGG	AGTCACCGTG TCAGTGGCAC	AGCTGGAACA TCGACCTTGT
1301	GCGGGGCGCT CGCCCCGCGA	GACCAGCGGC CTGGTCGCCG	GTGCATACCT CACGTATGGA	TTCCGGCGGT AAGGCCGCCA	GCTGCAAAGC CGACGTTTCG
1351	AGCGGCCTGT TCGCCGGACA	ATAGCCTGAG TATCGGACTC	CAGCGTTGTG GTCGCAACAC	ACCGTGCCGA TGGCACGGCT	GCAGCAGCTT CGTCGTGCAA
1401	AGGCACTCAG TCCGTGAGTC	ACCTATATTT TGGATATAAA	GCAACGTGAA CGTTGCACTT	CCATAAACCG GGTATTTGGC	AGCAACACCA TCGTTGTGGT
				EcoRI	
				~~~~~	
1451	AAGTGGATAA TTCACCTATT	AAAAGTGGAA TTTTCACCTT	CCGAAAAGCG GGCTTTTCGC	AATTCGGGGG TTAAGCCCCC	AGGGAGCGGG TCCCTCGCCC
1501	AGCGGTGATT TCGCCACTAA	TTGATTATGA AACTAATACT	AAAGATGGCA TTTCTACCGT	AACGCTAATA TTGCGATTAT	AGGGGGCTAT TCCCCGATA
				gIIIseq9 100.0%	
				=====	
1551	GACCGAAAAT CTGGCTTTTA	GCCGATGAAA CGGCTACTTT	ACGCGCTACA TGCGCATGT	GTCTGACGCT CAGACTGCGA	AAAGGCAAAC TTTCCGTTTG
				ClaI	
				~~~~~	
1601	TTGATTCTGT AACTAAGACA	CGCTACTGAT GCGATGACTA	TACGGTGCTG ATGCCACGAC	CTATCGATGG GATAGCTACC	TTTCATTGGT AAAGTAACCA
1651	GACGTTTCCG CTGCAAAGGC	GCCTTGCTAA CGGAACGATT	TGGTAATGGT ACCATTACCA	GCTACTGGTG CGATGACCAC	ATTTTGCTGG TAAAACGACC
1701	CTCTAATTCC GAGATTAAGG	CAAATGGCTC GTTTACCGAG	AAGTCGGTGA TTCAGCCACT	CGGTGATAAT GCCACTATTA	TCACCTTTAA AGTGGAAATT
1751	TGAATAATTT ACTTATTAAA	CCGTCAATAT GGCAGTTATA	TTACCTTCCC AATGGAAGGG	TCCCTCAATC AGGGAGTTAG	GGTTGAATGT CCAAC TTACA
1801	CGCCCTTTTG GCGGGAAAAC	TCTTTGGCGC AGAAACCGCG	TGGTAAACCA ACCATTGTGT	TATGAATTTT ATACTTAAAA	CTATTGATTG GATAACTAAC
1851	TGACAAAATA ACTGTTTTAT	AACTTATTCC TTGAATAAGG	GTGGTGTCTT CACCACAGAA	TGCGTTTCTT ACGCAAAGAA	TTATATGTTG AATATACAAC
1901	CCACCTTTAT GGTGGAATAA	GTATGTATTT CATACATAAA	TCTACGTTTG AGATGCAAAC	CTAACATACT GATTGTATGA	GCGTAATAAG CGCATTATTC

43/49

HindIII

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|      |             |            |            |             |             |
|------|-------------|------------|------------|-------------|-------------|
| 1951 | GAGTCTTGAT  | AAGCTTGACC | TGTGAAGTGA | AAAATGGCGC  | AGATTGTGCG  |
|      | CTCAGAACTA  | TTCGAACTGG | ACACTTCACT | TTTTACCGCG  | TCTAACACGC  |
|      |             | OGIII3     | 100.0%     |             |             |
|      |             | =====      |            |             |             |
| 2001 | ACATTTTTTTT | TGTCTGCCGT | TTAATGAAAT | TGTAAACGTT  | AATATTTTGT  |
|      | TGTAAAAAAA  | ACAGACGGCA | AATTACTTTA | ACATTTGCAA  | TTATAAAAACA |
| 2051 | TAAAATTTCGC | GTTAAATTTT | TGTTAAATCA | GCTCATTTTTT | TAACCAATAG  |
|      | ATTTTAAGCG  | CAATTTAAAA | ACAATTTAGT | CGAGTAAAAA  | ATTGGTTATC  |
| 2101 | GCCGAAATCG  | GCAAAATCCC | TTATAAATCA | AAAGAATAGA  | CCGAGATAGG  |
|      | CGGCTTTAGC  | CGTTTTAGGG | AATATTTAGT | TTTCTTATCT  | GGCTCTATCC  |
| 2151 | GTTGAGTGTT  | GTTCCAGTTT | GGAACAAGAG | TCCACTATTA  | AAGAACGTGG  |
|      | CAACTCACAA  | CAAGGTCAAA | CCTTGTTCTC | AGGTGATAAT  | TTCTTGCAACC |
| 2201 | ACTCCAACGT  | CAAAGGGCGA | AAAACCGTCT | ATCAGGGCGA  | TGGCCCACTA  |
|      | TGAGGTTGCA  | GTTTCCCGCT | TTTTGGCAGA | TAGTCCCGCT  | ACCGGGTGAT  |
| 2251 | CGAGAACCAT  | CACCCTAATC | AAGTTTTTTT | GGGTCGAGGT  | GCCGTAAAGC  |
|      | GCTCTTGGA   | GTGGGATTAG | TTCAAAAAAC | CCCAGCTCCA  | CGGCATTTTCG |
| 2301 | ACTAAATCGG  | AACCCTAAAG | GGAGCCCCCG | ATTTAGAGCT  | TGACGGGGAA  |
|      | TGATTTAGCC  | TTGGGATTTC | CCTCGGGGGC | TAAATCTCGA  | ACTGCCCTT   |
| 2351 | AGCCGGCGAA  | CGTGGCGAGA | AAGGAAGGGA | AGAAAGCGAA  | AGGAGCGGGC  |
|      | TCGGCCGCTT  | GCACCGCTCT | TTCTTCCCT  | TCTTTCGCTT  | TCCTCGCCCG  |
| 2401 | GCTAGGGCGC  | TGGCAAGTGT | AGCGGTCACG | CTGCGCGTAA  | CCACCACACC  |
|      | CGATCCCGCG  | ACCGTTCACA | TCGCCAGTGC | GACGCGCATT  | GGTGGTGTGG  |
| 2451 | CGCCGCGCTT  | AATGCGCCGC | TACAGGGCGC | GTGCTAGCCA  | TGTGAGCAAA  |
|      | GCGGCGCGAA  | TTACGCGGCG | ATGTCCCGCG | CACGATCGGT  | AACTCGTTT   |
| 2501 | AGGCCAGCAA  | AAGGCCAGGA | ACCGTAAAAA | GGCCGCGTTG  | CTGGCGTTTT  |
|      | TCCGGTTCGT  | TTCCGGTCCT | TGGCATTTTT | CCGGCGCAAC  | GACCGCAAAA  |
| 2551 | TCCATAGGCT  | CCGCCCCCCT | GACGAGCATC | ACAAAAATCG  | ACGCTCAAGT  |
|      | AGGTATCCGA  | GGCGGGGGGA | CTGCTCGTAG | TGTTTTTAGC  | TGCGAGTTCA  |
| 2601 | CAGAGGTGGC  | GAAACCCGAC | AGGACTATAA | AGATACCAGG  | CGTTTCCCCC  |
|      | GTCTCCACCG  | CTTTGGGCTG | TCCTGATATT | TCTATGGTCC  | GCAAAGGGGG  |
| 2651 | TGGAAGCTCC  | CTCGTGCGCT | CTCCTGTTCC | GACCCTGCCG  | CTTACCGGAT  |
|      | ACCTTCGAGG  | GAGCACGCGA | GAGGACAAGG | CTGGGACGGC  | GAATGGCCTA  |
| 2701 | ACCTGTCCGC  | CTTTCTCCCT | TCGGGAAGCG | TGGCGCTTTC  | TCATAGCTCA  |
|      | TGGACAGGCG  | GAAAGAGGGA | AGCCCTTCGC | ACCGCGAAAG  | AGTATCGAGT  |
| 2751 | CGCTGTAGGT  | ATCTCAGTTC | GGTGTAGGTC | GTTTCGCTCCA | AGCTGGGCTG  |
|      | GCGACATCCA  | TAGAGTCAAG | CCACATCCAG | CAAGCGAGGT  | TCGACCCGAC  |

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2801	TGTGCACGAA ACACGTGCTT	CCCCCGTTC GGGGGGCAAG	AGTCCGACCG TCAGGCTGGC	CTGCGCCTTA GACGCGGAAT	TCCGGTAACT AGGCCATTGA
2851	ATCGTCTTGA TAGCAGAACT	GTCCAACCCG CAGGTTGGGC	GTAAGACACG CATTCTGTGC	ACTTATCGCC TGAATAGCGG	ACTGGCAGCA TGACCGTTCGT
2901	GCCACTGGTA CGGTGACCAT	ACAGGATTAG TGTCCTAATC	CAGAGCGAGG GTCTCGCTCC	TATGTAGGCG ATACATCCGC	GTGCTACAGA CACGATGTCT
2951	GTTCTTGAAG CAAGAACTTC	TGGTGGCCTA ACCACCGGAT	ACTACGGCTA TGATGCCGAT	CACTAGAAGA GTGATCTTCT	ACAGTATTTG TGTCAATAAC
3001	GTATCTGCGC CATAGACGCG	TCTGCTGTAG AGACGACATC	CCAGTTACCT GGTCAATGGA	TCGGAAAAAG AGCCTTTTTTC	AGTTGGTAGC TCAACCATCG
3051	TCTTGATCCG AGAAGTAGGC	GCAAACAAAC CGTTTGTTTG	CACCGCTGGT GTGGCGACCA	AGCGGTGGTT TCGCCACCAA	TTTTTGTTTG AAAAACAAAC
3101	CAAGCAGCAG GTTTCGTCGTC	ATTACGCGCA TAATGCGCGT	GAAAAAAAGG CTTTTTTTTCC	ATCTCAAGAA TAGAGTTCTT	GATCCTTTTGA CTAGGAAACT
3151	TCTTTTCTAC AGAAAAGATG	GGGGTCTGAC CCCCAGACTG	GCTCAGTGGA CGAGTCACCT	ACGAAAACCTC TGCTTTTGAG	ACGTTAAGGG TGCAATTCCC
3201	ATTTTGGTCA TAAAACCAGT	GATCTAGCAC CTAGATCGTG	CAGGCGTTTA GTCCGCAAAT	AGGGCACCAA TCCCGTGGTT	TAAGTGCCTT ATTGACGGAA
3251	AAAAAAATTA TTTTTTTAAAT	CGCCCCGCCC GCGGGGCGGG	TGCCACTCAT ACGGTGAGTA	CGCAGTACTG GCGTCATGAC	TTGTAATTCA AACATTAAGT
3301	TTAAGCATTC AATTCGTAAG	TGCCGACATG ACGGCTGTAC	GAAGCCATCA CTTCGGTAGT	CAAACGGCAT GTTTGCCGTA	GATGAACCTG CTACTTGGAC
3351	AATCGCCAGC TTAGCGGTCTG	GGCATCAGCA CCGTAGTCGT	CCTTGTCGCC GGAACAGCGG	TTGCGTATAA AACGCATATT	TATTTGCCCA ATAAACGGGT
3401	TAGTGAAAAC ATCACTTTTG	GGGGGCGAAG CCCCGCTTC	AAGTTGTCCA TTCAACAGGT	TATTGGCTAC ATAACCGATG	GTTTAAATCA CAAATTTAGT
3451	AAACTGGTGA TTTGACCACT	AACTCACCCA TTGAGTGGGT	GGGATTGGCT CCCTAACCGA	GAGACGAAAA CTCTGCTTTT	ACATATTCTC TGTATAAGAG
3501	AATAAACCCCT TTATTTGGGA	TTAGGGAAAT AATCCCTTTA	AGGCCAGGTT TCCGGTCCAA	TTACCGGTAA AAGTGGCATT	CACGCCACAT GTGCGGTGTA
3551	CTTGCGAATA GAACGCTTAT	TATGTGTAGA ATACACATCT	AACTGCCGGA TTGACGGCCT	AATCGTCGTG TTAGCAGCAC	GTATTCACTC CATAAGTGAG
+1					
3601	CAGAGCGATG GTCTCGCTAC	AAAACGTTTC TTTTGCAAAG	AGTTTGCTCA TCAAACGAGT	TGGAAAACGG ACCTTTTGCC	TGTAACAAGG ACATTGTTCC
3651	GTGAACACTA CACTTGTGAT	TCCCATATCA AGGGTATAGT	CCAGCTCACC GGTCGAGTGG	GTCTTTTCATT CAGAAAGTAA	GCCATACGGA CGGTATGCCT
3701	ACTCCGGGTG TGAGGCCAC	AGCATTCATC TCGTAAGTAG	AGGCGGGCAA TCCGCCCGTT	GAATGTGAAT CTTACACTTA	AAAGGCCGGA TTTCCGGCCT

3751 TAAAACTTGT GCTTATTTTT CTTTACGGTC TTTAAAAAGG CCGTAATATC  
ATTTTGAACA CGAATAAAAA GAAATGCCAG AAATTTTTC CCGCATTATAG

3801 CAGCTGAACG GTCTGGTTAT AGGTACATTG AGCAACTGAC TGAAATGCCT  
GTCGACTTGC CAGACCAATA TCCATGTAAC TCGTTGACTG ACTTTACGGA

3851 CAAAATGTTT TTTACGATGC CATTGGGATA TATCAACGGT GGTATATCCA  
GTTTTACAAG AAATGCTACG GTAACCCTAT ATAGTTGCCA CCATATAGGT

3901 GTGATTTTTT TCTCCATTTT AGCTTCCTTA GCTCCTGAAA ATCTCGATAA  
CACTAAAAAA AGAGGTAAAA TCGAAGGAAT CGAGGACTTT TAGAGCTATT

3951 CTCAAAAAAT ACGCCCGGTA GTGATCTTAT TTCATTATGG TGAAAGTTGG  
GAGTTTTTTT TGCGGGCCAT CACTAGAATA AAGTAATACC ACTTTCAACC

4001 AACCTCACCC GACGTCTAAT GTGAGTTAGC TCACTCATTA GGCACCCAG  
TTGGAGTGGG CTGCAGATTA CACTCAATCG AGTGAGTAAT CCGTGGGGTC

4051 GCTTTACACT TTATGCTTCC GGCTCGTATG TTGTGTGGAA TTGTGAGCGG  
CGAAATGTGA AATACGAAGG CCGAGCATAC AACACACCTT AACACTCGCC

M13 Reverse primer 100.0%

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4101 ATAACAATTT CACACAGGAA ACAGCTATGA CCATGATTAC GAATT  
TATTGTAAAA GTGTGTCCTT TGTCGATACT GGTACTAATG CTAA

## Figure 15

MS-GPC-1:

VH

QVQLKESGPALVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDATYY  
CARQYGHRGGFDHWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDFNE  
SVFGGGTKLTVLG

MS-GPC-6

VH

EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYAMSWVRQAPGKGLE  
WVSAISGSGGSTYYADSVKGRFTISRDN SKNTLYLQMNSLRAEDTAVY  
YCARGYGRYSPDLWGQGTLVTVSS

VL

DIVLTQSPATLSLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRLLI  
YGASSRATGVPARFSGSGSGTDFTLTISSELPEDFAVYYCQQYSNLPF  
TFGQGGTKVEIKRT

MS-GPC-8

VH

QVQLKESGPALVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDATYY  
CARSPRYRGAFDYWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDMPQ  
AVFGGGTKLTVLG

MS-GPC-10

VH

QVQLKESGPALVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDTATYY  
CARQLHYRGGFDLWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDLTM  
GVFGGGTKLTVLG

MS-GPC-8-6

VH

QVQLKESGPALVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDTATYY  
CARSPRYRGAFDYWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDYDH  
YVFGGGTKLTVLG

MS-GPC-8-10

VH

QVQLKESGPALVKPTQTLTLCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDATYY  
CARSPRYRGAFDYWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDLIRH  
VFGGGTKLTVLG

MS-GPC-8-17

VH

QVQLKESGPALVKPTQTLTLCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDATYY  
CARSPRYRGAFDYWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDFSV  
YVFGGGTKLTVLG

MS-GPC-8-27

VH

QVQLKESGPALVKPTQTLTLCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDATYY  
CARSPRYRGAFDYWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSSSNIGSNYVSWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDMNV  
HVFGGGTKLTVLG

MS-GPC-8-6-13

VH

QVQLKESGPALVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDTATYY  
CARSPRYRGAFDYWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSESNIGANYVTWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDYDH  
YVFGGGTKLTVLG

MS-GPC-8-10-57

VH

QVQLKESGPALVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDTATYY  
CARSPRYRGAFDYWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSESNIGNNYVQWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDLIRH  
VFGGGTKLTVLG

MS-GPC-8-27-41

VH

QVQLKESGPALVKPTQTLTLTCTFSGFSLSTSGVGVGWIRQPPGKALE  
WLALIDWDDDKYYSTSLKTRLTISKDTSKNQVVLTMNMDPVDTATYY  
CARSPRYRGAFDYWGQGTLVTVSS

VL

DIVLTQPPSVSGAPGQRVTISCSGSESNIGNNYVQWYQQLPGTAPKLLI  
YDNNQRPSGVPDRFSGSKSGTSASLAITGLQSEDEADYYCQSYDMNV  
HVFGGGTKLTVLG



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US01/15625

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7)- :A61K 39/395, 44

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 138.1, 138.1, 141.1, 143.1, 144.1, 152.1, 153.1, 155.1, 172.1, 173.1, 174.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CANCERLIT, WEST  
search terms antibodies, apoptosis, HLA-DR**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	DUEYMES et al. Anti-endothelial cell antibody binding causes apoptosis of endothelial cells. Arthritis & Rheumatism. September 1997, Vol.40, page S103. See abstract.	1 --- 109, 110
X --- Y	KIM et al. Altered expression of the genes regulating apoptosis in multidrug resistant human myeloid leukemia cell lines overexpressing MDR1 or MRP gene. 1997, Vol.11, pages 945-950. See abstract.	1 --- 109,110
X --- Y	ISHIZUKA et al. Antitumour activity of murine monoclonal antibody NCC-ST-421 on human cancer cells by inducing apoptosis. Anticancer Research. July-August 1998, Vol.18, pages 2513-2518. See abstract.	1 --- 109-110

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"G" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

29 JULY 2001

Date of mailing of the international search report

15 AUG 2001

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## INTERNATIONAL SEARCH REPORT

International application No.

PC17US01/15625

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	GHAHREMANI et al. Activation of fas ligand/receptor system kills ovarian cancer cell lines by apoptotic mechanism. Gynecological Oncology. August 1998, Vol.70, pages 275-281. See abstract.	1 --- 109, 110
X --- Y	HENSEL et al. Characterization of glycosylphosphatidylinositol-linked molecule CD55/decay-accelerating factor as the receptor for antibody SC-1-induced apoptosis. Cancer Research. 15 October 1999, Vol.59, pages 5299-5306. See abstract.	1 --- 109,110
X --- Y	NAKAMURA et al. Apoptosis induction of the human lung cancer cell line in multicellular heterospheroids with human antigangliosides GM2 monoclonal antibody. Cancer Research. 15 October 1999, Vol.59, pages 5323-5330. See abstract.	1 --- 109, 110
X --- Y	WALLEN-OHMAN et al. Antibody-induced apoptosis in a human leukemia cell line is energy dependent. Cancer Letters. 10 December 1993, Vol.75, pages 103-109. See abstract.	1 --- 109, 110
X --- Y	MYSLER et al. The apoptosis-1/Fas protein in human systemic lupus erythematosus. Journal of Clinical Investigation. March 1994, Vol.93, pages 1029-1034. See abstract.	1 --- 109, 110
X --- Y	ACKERMAN et al. Induction of apoptotic or lytic cell death in an ovarian adenocarcinoma cell line by antibodies generated against a synthetic N-terminal extracellular domain gonadotropin-releasing hormone receptor peptide. Cancer Letters. 30 June 1994, Vol.81, pages 177-184. See abstract.	1 --- 109, 110
X --- Y	ERAY et al. Cross-linking of surface IgG induces apoptosis in bel-2 expressing human follicular lymphoma line of mature B cell phenotype. International Immunology. December 1994, Vol.6, pages 1817-1827. See abstract.	1 --- 109, 110
X --- Y	NAKAMURA et al. Apoptosis in human hepatoma cell line induced by 4,5-didehydro geranylgeranoic acid via down-regulation of transforming growth factor-alpha. Biochemical and Biophysical Research Communications. 06 February 1996, Vol.219, pages 100-104. See abstract.	1 --- 109, 110
X --- Y	VOLLMERS et al. Apoptosis of stomach carcinoma cells induced by a human monoclonal antibody. Cancer. 15 August 1995, Vol.76, pages 550-558. See abstract.	1 --- 109, 110
X --- Y	HATA et al. Fas/Apo-1 (CD95)-mediated and CD95-independent apoptosis of malignant plasma cells. Leukemia and Lymphoma. December 1996, Vol.24, pages 35-42. See abstract.	1 --- 109, 110

## INTERNATIONAL SEARCH REPORT

Internal application No.  
PCT/US01/15625

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	HIGASHIGAWA et al. FK506 inhibits anti-IgM antibody-induced apoptosis and 17 kD endonuclease activity in the human B-cell line, MBC-1, established from Burkitt's lymphoma. British Journal of Haematology, December 1997, Vol.99, pages 908-913. See abstract.	1 --- 109, 110
X --- Y	MASUDA et al. Dual action of CD30 antigen: anti-CD30 antibody induced apoptosis and interleukin-8 secretion in Ki-1 lymphoma cells. International Journal of Hematology, April 1998, Vol.67, pages 257-265. See abstract.	1 --- 109, 110
X --- Y	HAYAKAWA et al. A short peptide derived from the antisense homology box of Fas ligand induces apoptosis in anti-Fas antibody-insensitive human ovarian cancer cells. Apoptosis. February 2000, Vol.5, pages 37-41. See entire document.	1 --- 109, 110
X --- Y	VIDOVIC et al. Selective apoptosis of neoplastic cells by the HLA-DR-specific monoclonal antibody. Cancer Letters. 19 June 1998, Vol.128, pages 127-135. See entire document.	1-5, 7-10, 18, 19, 21 ----- 6, 14, 15, 38, 66, 68, 69, 70, 109-114
X --- Y	LEE et al. HLA-DR-Triggered Inhibition of Hemopoiesis Involves Fas/Fas Ligand Interactions and Is Prevented by c-kit Ligand. Journal of Immunology. 01 October 1997, Vol.159, pages 3211-3219. See entire document.	1-5, 7-10 ----- 6, 14, 15, 38, 69 109-114
X --- Y	LEE et al. HLA-DR-Mediated Signals for Hematopoiesis and Induction of Apoptosis Involve But Are Not Limited to a Nitric Oxide Pathway. Blood. 01 July 1997, Vol.90, pages 217-225. See entire document.	1-5, 7-10 ----- 6, 14, 15, 38, 69 109-114
X --- Y	McDEVITT et al. Monoclonal anti-Ia antibody therapy in animal models of autoimmune disease. Ciba Foundation Symposium. 1987, Vol.129, pages 184-193. See entire document.	1-5, 7-10, 66, 68, 70 ----- 6, 14, 15, 38, 69
Y	HARRISON et al. Screening of Phage Antibody Libraries. Methods in Enzymology. 1996, Vol.267, pages 83-109. See entire document.	6, 69
Y	ROOS et al. Establishment and characterization of a human EBV-negative B cell line. Leukemia Research. 1982, Vol.6, pages 685-693. See abstract.	14, 15

## INTERNATIONAL SEARCH REPORT

International application No.

PC1/US01/15625

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TOSI et al. Immunochemical definition of the new dr specificity 8WDRw13. Immunological Communications. 1981, Vol.10, pages 275-292. See abstract.	14, 15

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US01/15625

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/180.1, 133.1, 138.1, 141.1, 143.1, 144.1, 152.1, 153.1, 155.1, 172.1, 173.1, 174.1

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US01/15625

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 11-13,24-37,39-65,71-108  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.